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FINAL REPORT

on

STUDIES ON THE BIODEGRADATION OF ORDNANCE- RELATED HAZARDOUS WASTE

PHASE II: SOIL DEGRADATION KINETICS

**CATEGORY: MANUFACTURING OF ORDNANCE AND ORDNANCE RELATED
COMPONENTS AND CHEMICALS**

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EXECUTIVE SUMMARY

Aerobic degradation studies were conducted for four nitrate ester compounds: Nitroglycerine or Glycerol Trinitrate, Propylene Glycol dinitrate (PGDN), Triethylene glycol dinitrate (TEGDN) and Trimethyloethane trinitrate (TMETN). Soil microcosm reactors were designed, assembled and operated to acclimate the soil microbiota to a mixture of the four selected nitrate ester compounds. Uncontaminated forest soil with low organic carbon was used in all studies. Carbon dioxide evolution measurements were made to monitor the acclimation process. Initially, the carbon dioxide evolution in the contaminated microcosm reactor was higher than the control (uncontaminated) microcosm reactor. However, after 42 days, the rate of carbon dioxide evolution in the two microcosm systems became nearly equal indicating the acclimation period. Soil from the contaminated microcosm reactor was used in all subsequent biokinetic studies. Adsorption and desorption studies were conducted using soil slurry systems. Negligible adsorption of the nitrate ester compounds was measured for the selected soil. Slurry biokinetic studies were conducted using an electrolytic respirometer wherein cumulative oxygen uptake was monitored. All four compounds degraded at an initial concentration of 100 mg/L. However, at an initial concentration of 200 mg/L, all four compounds were partially mineralized and exhibited inhibition effects. Inhibition effects were experimentally observed by degrading a mixture of the compound with an easily degradable compound, aniline. Biokinetic parameters were obtained by fitting the cumulative oxygen uptake curve to a mathematical model based on the Monod equation. The rate of biodegradation observed was in the following order: GTN > TEGDN > PGDN > TMETN. Cumulative carbon dioxide evolution measurements were also made to determine the extent of mineralization. At 100 mg/L, all four compounds were completely mineralized eventually. Partial mineralization was observed at 200 mg/L due to toxicity effects to the soil microbiota.

Results reported here demonstrate that soil microbiota can be acclimated to all four nitrate ester compounds at a concentration of 100 mg/L. This indicates that soil contaminated with nitrate ester compounds can be treated successfully in a soil slurry reactor.

INTRODUCTION

The molecular processes involved in microbial and enzymatic degradation are being exploited by agencies of the U.S. Department of Defense in diverse ways. Biodegradation often provides an attractive or adjunct to conventional methods of restoring contaminated soils or groundwater, or treating wastewater containing hazardous chemicals. In this report, biodegradation refers to the use of microorganisms or enzymes to break down chemical compounds.

In 1980, Congress enacted legislation to identify, finance, and monitor cleanup of the nation's most serious hazardous waste dumps. Currently, DOD has 739 sites in need of remediation, and cleanup costs are estimated at five to 10 billion U.S. dollars. The primary hazardous wastes found on military installations are fuels, cleaning solvents, propellants, explosives, etc. Specifically, this report is addressing ordnance-related hazardous chemicals, such as Nitroglycerine, Glycerol trinitrate, Propylene Glycol Dinitrate, Triethylene glycol dinitrate, and Tri methyl ethane trinitrate.

Virtually all conventional technologies for land reclamation have proved to be unsatisfactory. For example, landfilling is not a permanent solution, and costs are increasing dramatically (in the United States, from US \$10 to US \$250 per ton in the last five years). Incineration produces toxic air pollutants, and many organic compounds are difficult to burn. In situ solidification (chemical fixation), capping, and vitrification are largely unproved, costly, and aesthetically objectionable technologies. Volatile organics in groundwater have been removed via strategically located slotted wells and by air stripping where contaminated groundwater is pumped through large packed-bed towers; both are costly, labor-intensive solutions and require further treatment of the contaminated air stream that is generated.

Furthermore, conventional methods for treatment of wastewater are expensive and require further treatment of the gas and solid phases that are generated. For example, conventional activated sludge treatment requires large-scale plant size, with a retention time in the range of 6-9 days, and generates contaminated air phase and sludges requiring further treatment.

Bioprocessing or biodegradation often provides a low-technology, permanent, inexpensive, effective, nonpolluting alternative for land reclamation and treatment of industrial effluents [1]. Microbes have evolved or can be adapted to degrade virtually any toxic organic chemical. Hydrocarbons, a major class of military wastes, are particularly susceptible to biodegradation. Biodegradation is effective over a range of environmental conditions, and for a wide variety of contaminants. Often, bioprocesses can be integrated with conventional technologies, resulting in efficient, multicomponent systems.

PROJECT OBJECTIVES

The specific objective of this project was to determine aerobic biodegradation kinetics and toxicity levels in soil systems for the following compounds: Nitroglycerine, Propylene Glycol dinitrate (PGDN), Triethylene glycol dinitrate (TEGDN) and Trimethyloethane trinitrate (TMETN).

BACKGROUND

The microbial degradation of glycerol trinitrate (GTN) has been reported in the literature [2]. The degradation process proceeds through a series of successive denitration steps through glycerol dinitrate and glycerol mononitrate isomers, with each succeeding step proceeding at a slower rate.

In addition to direct microbial degradation, chemical treatments of GTN have been developed in order to desensitize waste streams, resulting in the disappearance of glycerol tri-, di-, and mononitrates, but with the corresponding formation of glycidol and glycidyl nitrate [3]. These products contain a highly reactive epoxide moiety that tends to confer mutagenic properties.

The chemical by-products, glycidol and glycidyl nitrate, have also been studied to determine their biodegradability. The pathway from glycidyl nitrate to glycerol 1-mononitrate to glycerol proceeds more slowly with each succeeding step. The steps from glycidol to glycerol and glycidyl nitrate to glycerol 1-mononitrate occur spontaneously in aqueous solutions but appear to be accelerated (directly or indirectly through secondary effects) by microbial activity [3].

Glycidol and glycidyl nitrate tested positive in the Ames test screening for mutagenicity, whereas the transformation product glycerol-1-mononitrate tested negative [3]. Both glycerol trinitrate and glycerol-1-mononitrate have been shown to be toxic to mammals [4].

Propylene glycol dinitrate (PGDN), diethylene glycol dinitrate (DEGN), triethylene glycol dinitrate (TEGDN), and trimethylolthane trinitrate (TMETN) undergo microbial transformation via successive denitration steps, leading to the formation of the corresponding glycols: propylene glycol (PG), diethylene glycol (DEG), triethylene glycol (TEG), and trimethylolethane glycol (TMEG) (refer Figure 3)[5].

The degradation of the resulting glycols at concentrations of 100 mg per liter has also been assessed [6]. Rates of degradation were as follows: PG > DEG > TEG > TMEG, from high to low, although degradation appeared to be due to a combination of biological and non-biological processes.

PG, DEG, and TEG present minimal toxicity and carcinogenic hazards. PG is the least toxic of the glycols and is commonly used in pharmaceutical, cosmetic, and food applications. DEG and TEG are slightly toxic; repeated large doses are needed for toxicity [7]. TMEG had negative test results in AMES test screening for mutagenicity [6].

A generic biological treatment concept for wastewaters contaminated with hazardous Nitrate

Esters is as follows:

INFLUENT ----> CHEMICAL PRETREATMENT ---->
 (if required)
 ANAEROBIC
 DENITRIFICATION ----> AEROBIC TREATMENT ----> EFFLUENT

Mixed cultures were used in many of the studies reported here. The ability of microorganisms to biotransform this class of compounds is apparently ubiquitous in nature. The use of acclimatized cultures from environments previously exposed to the compounds under study perhaps would have accelerated some of the initial rates of transformation observed; however, once acclimatized, random environmental microbial inocula were capable of transforming the compounds studied.

Recent studies report that microbial transformations of some of organo-nitro compounds were dependant on the availability of primary growth substrates [8]. The biotransformation of recalcitrant chemicals with some of primary carbon source has been referred to as cometabolism. Similarities have been reported on the degradation of nitroglycerin in sewage and lake water systems [9,10,11]. These recalcitrant chemicals are non-growth substrates which do not supply sole carbon and energy source to microbiota. Therefore, bacterial growth yield from the cellular division does not occur in the degradation of these chemicals. The potential of using these organo-nitro compounds as available sole carbon and energy sources before the denitration phase has not yet been fully studied.

Aerobic degradation of nitrate ester and aromatic compounds using pure and mixed cultures have been studied by many researchers [12, 13, 14, 15, 16]. However, little information is available on the biokinetics of nitrate ester compounds in aqueous or soil slurry systems.

CHARACTERIZATION OF SOIL

Soils found in different regions of the world obviously are diverse in composition based on climate, geology and other factors. Because of the diverse nature of soil properties even within a particular region, it is necessary to characterize any soil being used in soil studies based on several parameters. This is important so that comparisons between different research results can be made. Some basic properties of soils which can vary from system to system are: soil particle size distribution, soil surface area, water and air permeability, moisture content, organic matter content and microbial content of the soil. The following will detail experiments planned to help characterize the soil being used in these studies.

Moisture Content

The moisture content of the soil may be very easily found. A small soil sample was brought into the laboratory from the field and immediately weighed on a tared watch glass. The sample was then

placed in an oven set at 110 degrees Celsius for a few hours, and then weighed once again. The sample was dried in the oven and re-weighed repeatedly until a constant dry weight was found. The original moisture content of the soil was then easily calculated according to the following simple formula:

$$\text{Moisture \%} = [(\text{Original mass}) - (\text{Dry mass})] / (\text{Original mass})$$

Particle Size Distribution

Particle size distribution is perhaps the most important characteristic used to classify soils. The pipette method of particle size analysis described by Day [17] will be closely followed in this research. This method is based on the knowledge that particles settle at different rates through a stagnant medium based on their relative sizes. The Stokes equation gives the following relationship between a particle's settling velocity and its size:

$$v = \frac{d^2 g (\rho_s - \rho_l)}{18\mu}$$

where v = the particle settling velocity

d = the particle diameter

g = gravitational acceleration

ρ_s = particle density

ρ_l = settling media density

μ = liquid (settling media) viscosity

A system was set up according to the specifications set forth by Day [17] and as shown in Figure 1. A 7 % soil suspension was prepared and placed in a 1.0 liter graduated cylinder along with 250.0 mL. of a 10 % solution of sodium metaphosphate (dispersing agent), and stirred for several minutes. Using the above equation, the time required for all particles greater than a given particle size to settle through 80% of the container depth was calculated.

The cylinder was then placed on a level table and a timer was started. When the previously determined time had passed, a pipette was carefully lowered into the container and all of the liquid/suspension down to 80 % of the container depth was removed and placed in a glass bottle. Distilled water was then added back into the container to the original fill line, and the suspension well mixed again, and the exact procedure was repeated. This was done until the fraction being siphoned off no longer contained soil particles. At this time, the discard bottle was placed in an oven overnight (approximately 110 degrees Celsius) to drive off all of the water present. The weight of soil left was an indication of the fraction of the entire soil sample which had a diameter smaller than the chosen value.

A new (larger) diameter was then chosen, and a new time was calculated, and the entire process was repeated. The soil was split roughly into three fractions based on these analyses, corresponding to the clay (less than 2 microns), silt (greater than 2 microns but less than 20 microns), and sand (greater than 20 microns but less than 2000 microns) fractions of the soil. The soil was then simply classified based on

the percentage of clay, silt, and sand it contains.

Total Carbon Content Determination

Carbon occurs in soils in both organic and inorganic (carbonate) form. Allison, Bollen, and Moodie [18] discuss the background for the analysis of total carbon in soils and discuss both wet and dry combustion techniques for this analysis. A modified version of their wet combustion method was used in the present research due to its relative simplicity. Figure 2 shows the apparatus to be used. Unit A is a simple laboratory heating plate with magnetic stirring capability. Unit B is a 125 mL. flat-bottomed round flask, fit with an appropriate ground glass fitting to allow CO₂-free air to be bubbled through the reactor contents. Units C, D, and E are glass bottles which will be used to remove unwanted constituents of the combustion gas. These bottles will be fit with ground glass fittings identical to those for the reactor flask. Unit F is a high efficiency gas absorption bottle, which will be used to absorb CO₂ from the combustion gas. Finally, a standard laboratory vacuum pump was used to help move the combustion gas through the system.

A ten gram sample of finely crushed, dried soil was placed in the reaction flask with 10.0 mL. of water, 80.0 mL. of a 60:40 mixture of concentrated sulfuric acid and 85% Phosphoric acid, and about 3.5 grams of potassium dichromate. Boiling this mixture at about 200 degrees Celsius produced a gas made up mainly of carbon dioxide (CO₂) gas, chlorine gas, and water vapor. Bottles C and D were filled, respectively, with a 50 % solution of potassium iodide and a saturated aqueous solution of silver sulfate, which will both absorb any chlorine in the combustion gas. Bottle E was filled with magnesium perchlorate powder which will remove any water vapor from the gas. Finally, an aqueous solution of potassium hydroxide (KOH) with a pH of about 12 was used to absorb all of the CO₂ produced. The amount of CO₂ produced was analyzed based on the change in pH of the KOH trapping solution [19,20]. This information was used to determine the total amount of carbon in the soil sample according to the following equation:

$$\text{Total carbon \%} = (\text{Grams CO}_2 / \text{Grams dry soil}) \times 0.2727 \times 100$$

Soil Carbonate Content Determination

The method for the determination of soil carbonate was very similar to the total carbon content determination, with the CO₂ produced by a combustion procedure being the determining factor. The same apparatus as used above was used with only slight modifications. In this case, the digestion acid mixture contained approximately 5 % FeSO₄, which acts as an antioxidant and prevents organic carbon from being converted to CO₂. The digestion acid mixture, as recommended by Allison and Moodie [21], is made up by dissolving 92.0 grams of FeSO₄·7H₂O along with 57 mL. of concentrated sulfuric acid in 1.0 liter of water. About 80.0 mL. of this mixture was boiled for approximately 30 minutes with 10.0 grams of soil. The same traps and CO₂ analysis as used in the total carbon analysis were used. The

above formula was applied to calculate the amount of soil carbonate in the soil.

Organic Matter Content

The amount of organic carbon in soil can be found by methods very similar to those used above for the determination of total carbon and soil carbonate [22]. More simply, however, the amount of organic carbon in a soil can be found simply by subtraction by noting that the total carbon in the soil is made up only of soil carbonate and organic carbon. Thus, if the total carbon and carbonate analyses are both completed first, the amount of organic carbon in the soil will be equal to the amount of total carbon in the soil minus the amount of soil carbonate.

Obviously, the organic matter in a soil is not made up only of carbon. Some researchers have suggested a factor of about two relates the amount of organic matter in soil to the amount of organic carbon in the soil [23]. More often, only organic carbon content is reported and total organic matter is usually not mentioned.

Soil Surface Area Determination

The physical and chemical properties of a soil depend greatly on the soil surface area. A common method of analysis for the surface area of a soil is based on the knowledge that the chemical ethylene glycol is adsorbed by soil particles to form a monomolecular layer on the soil surface. To determine the surface area of the soil used in these studies, 1.1 grams of soil was evenly spread over the bottom of a tared aluminum box. It was then placed in a vacuum desiccator over approximately 250 grams of P_2O_5 and dried to a constant weight. The soil sample was then warmed slightly and wetted with ethylene glycol. Finally, the box was placed over 120 grams of $CaCl_2$ -glycol solvate and dried to a constant weight in a vacuum desiccator at 25 degrees Celsius. The $CaCl_2$ -glycol solvate was prepared by adding 100 grams of dried $CaCl_2$ (at 210 degrees Celsius) to 20 grams of glycol in a 400 mL beaker and mixing well. The surface area of the soil was then calculated simply according to the following equation:

$$A = \frac{W_g}{(0.000311W_s)}$$

where: A is the specific surface area in square meters per gram
 W_s is the dried weight of the soil sample
 and W_g is the weight of glycol retained.

Pore size Distribution and Surface Area

The pore size distribution, average pore size, pore volume and surface area (BET) were determined by nitrogen adsorption and desorption porosimetry (Micromeritics, ASAP 2000), and the results are shown below:

BET specific surface area(m^2/g) 20.2691

BET void volume (cc/g) 0.029390
BET average pore diameter (Å) 57.9991

Results and Discussion

The results of soil characterization are summarized in Table 1. The soil used in this study had low organic carbon (< 1%) and had an average particle size distribution in the range of 300-600 μm . The carbonate content of the soil was negligible. Additional soil obtained from Indian Head, MD had very similar characteristics and hence was not pursued in this study.

ANALYTICAL METHODOLOGY

Chemicals

Four nitrate ester compounds: nitroglycerin (NG), propylene glycol dinitrate (PGDN), trimethyloethane trinitrate (TMETN) and triethyleneglycol dinitrate (TEGDN), were obtained from Naval Surface Warfare Center, Indian Head Division, MD. The chemicals for HPLC analysis were purchased as reagent grade from Fisher Scientific, Cincinnati, OH and Sigma Chemical Co., St. Louis, MO.

Analytical Method

High-performance liquid chromatography was used to quantitate the four parent nitrate ester compounds. Analyses were performed on a Waters Associates (Milford, MA) HPLC equipped with a M45 solvent delivery pump with a Waters U6K injector, a M481 variable wavelength detector monitoring at 196 nm. All analyses were run on a μ Bondapak phenyl reverse-phase stainless-steel column, 8 mm by 10 cm with a 10 μm particle size. The mobile phase used was a mixture of 64% water/36% acetonitrile at a flowrate of 1.5 mL per minute. The composition of the mobile phase and the retention times of all the compounds is given in Table 2. Figures 3 through 6 show the standard curves for NG, PGDN, TEGDN and TMETN respectively. The standard curves are linear for concentrations less than 100 mg/L.

STUDIES ON ADSORPTION/DESORPTION OF NITRATE ESTER COMPOUNDS IN SOIL SLURRY SYSTEMS

Adsorption of compounds in soil has been extensively studied in the literature since it directly affects the bioavailability of the compound for aqueous phase biodegradation. Considerable work has been conducted on the partitioning of neutral hydrophobic organic compounds to soils and sediments. Generally, the magnitude of partitioning to the natural organic carbon of a soil or sediment can be related to a chemical's octanol-water partition coefficient or its water solubility. Karickhoff [24] derived a semi-empirical relationship for the partitioning at equilibrium of PAH compounds to soils and sediments. For even moderately hydrophobic compounds ($K_{ow} > 4.0$), Karickhoff's empirical relationship implies that these compounds will partition significantly to sediments or soils that contain significant organic carbon.

thus reducing their availability to microorganisms.

Adsorption of organics from solution at a solid-liquid interface is a complex and imperfectly understood phenomenon. The main physicochemical forces thought to be responsible for adsorption are: London-van der Waals, coulombic-electrostatic-chemical, hydrogen bonding, ligand exchange-anion penetration-coordination, chemisorption, dipole-dipole or orientation energy, induction or dipole-induced dipole and hydrophobic effect.

Although soil is highly heterogeneous from the microscopic point view, generally, the adsorbent phase in soil constitutes mineral and organic matter. Chiou [25] reviewed previous research and argued that when the organic content is high enough, sorption of nonionic organic compounds by soil in aqueous systems is mainly controlled by the soil organic matter content. In this case, the partition coefficient may be related to water solubility. The diverse sorption characteristics with high and low organic carbon matter soils can be reconciled by the postulate that the soil behaves as a dual sorbent, in which the mineral matter functions as a conventional solid adsorbent and the organic matter as a partition medium. The inability of the soil mineral fraction to adsorb nonionic organic compounds from water is attributed to the strong dipole interaction of minerals with water, which excludes the organic compounds from this portion of the soil.

Experimental procedure for Adsorption Studies

20 g soil was placed in a 125 ml glass bottle. Various concentrations of the four nitrate ester compounds: 10, 25, 50, and 100 mg/L were used. The volumes of the total solution added to each bottle was 100 ml in order to maintain a minimum head space. Blanks containing only nitrate ester solution allowed the measurement of volatilization losses, if any. A separate study was carried out to determine the adsorption-desorption of the compounds by the glass bottle containers and was found to be negligible for all the containers used in this study. In order to prevent degradation in solution, 1 ml HgCl_2 saturated solution was added to each bottle. All adsorption-desorption experiments were conducted in a fume hood, so the temperature of adsorption-desorption was controlled at 24°C.

The bottles were stirred using a magnetic stirrer for different time periods (4, 8, 16, 24, 48 hours). The contents were centrifuged in a refrigerated centrifuge. The supernatant was filtered using a 0.45 μm silver membrane and placed in a 50 ml sample vial for analysis.

Experimental Procedure for Desorption Studies

For determining desorption isotherms, each medium was first subjected to adsorption using the above adsorption procedure. After equilibrium was attained, the free supernatant was discarded and replaced with distilled deionized water. These samples were then treated the same way as for adsorption studies although the time taken to achieve desorption equilibrium was significantly longer.

Results and Discussion

No significant adsorption or desorption effects were measured for the selected soil and the four nitrate ester compounds. Changes in liquid phase concentration as a function of time were less than 5%

of the initial concentration and hence it was concluded that the nitrate ester compounds do not adsorb significantly in the soil phase.

Preliminary studies conducted with another soil sample obtained from Naval Surface Warfare Center, Indian Head Division, MD also produced very similar results. Hence, detailed investigation of this soil was not conducted.

STUDIES WITH SOIL MICROCOSM REACTORS FOR ACCLIMATING SOIL MICROBIOTA

Microcosm systems are being successfully used as laboratory simulations of contaminated soil sites to assess the biodegradability of organic pollutant compounds by microbiota in intact soil-cores; to determine biodegradation rates and extent of biodegradation; to acclimate soil microorganisms to the freshly polluted soil matrices; and to evaluate the risks of genetically engineered microorganisms to the environment. Studies were reported on the toxic effects of pollutants (PCP) on the mineralization of acetate in subsoil microcosms. Exponential growth kinetics with acetate were established for the non-inhibiting and inhibiting concentrations of PCP. The model predicts that slow-growing microbiota which are vital to the functioning of the ecosystem, would recover only very slowly from the toxic effects of environmental pollution. The fate of methylparathion and the development of biological communities were examined as a function of inorganic nutrient enrichment, inorganic nutrients plus glycerol and presence of sediments in eight-compartment continuous flow channel microcosms.

A microcosm study on biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons was attempted in a methanogenic aquifer material. Microcosms containing intact soil-cores have served as a tool for assessing the risks of the release of genetically engineered microorganisms to the environment. Intact soil-core microcosms were compared to growth chambers, field lysimeters and field plots. Microcosms incubated at ambient temperature in the laboratory and the growth chambers were similar to those in the field with respect to survival of and colonization of the rhizosphere by the introduced *Pseudomonas* sp.

The soil microcosm reactor is made of glass and its frame is supported by stainless steel panel. The schematic diagram is shown in Figure 7. Twenty inches of length, twelve inches of height, and twelve inches of width is used for this reactor dimensions. 5 mm thick glass lid is used to cover the reactor, and eight joints were used for clamping the top lid so that the whole system was closed tightly. Glass cover has six stainless steel liquid atomizing sprayer (Spraying Systems Inc., Chicago, IL) at an equal distance. This liquid atomizing sprayer sprays the nutrient solution on top of the soil bed at a pre-determined time period using automatic timer. The automatic timer is directly connected to the stainless steel solenoid valve and controls the openings a day. The container used for the nutrient storage and effluent recycle storage is made of stainless steel (Chapin sprayer, New York). The nutrient storage tank is placed in the refrigerator. Two filters of 2 μ m pore size are installed in front of the solenoid and right

after the effluent comes out of the microcosm reactor to remove the microorganism. Two holes on the left and right side of the glass reactor are made for the inlet and outlet of flow regulated air. The air supplies the oxygen to the indigenous microorganism in the soil as well as pushing the head space gas out of the microcosm reactor to the potassium hydroxide trapping bottles to remove the carbon dioxide which is the product of the biodegradation activity in the soil bed. Titration method is adapted to measure the carbon dioxide concentration for calculating the mass balance. Since this work is only done in the surface soil (vadose zone), the air in the head space is assumed to diffuse to the very bottom of the soil bed. The dissolved oxygen meter simultaneously checks the oxygen concentration level in the effluent. All connecting lines used for the reactors are made of tygon or teflon tubing, and all containers are made of stainless steel or glass, or otherwise specified.

Soil Cube Sampling.

To simulate the natural soil infiltration situation, the integrity of the soil cube should not be disturbed. The depth of a soil cube dictates the method of excavation. The pits and trenches may be excavated by hand or by conventional earth-excavating equipment. Accessible borings may be drilled with special-purpose drilling rigs. For the research related to surface soil, the procedure for obtaining the block or cube soil samples (Department of the Army, 1972) is applied. The surface of the area to be sampled is trimmed smooth and nearly level. In general, a square column of soil 2 inches smaller than the inside dimensions of the box is carefully trimmed to a depth 1/2 inches less than the inside depth of the box, using a knife, shovel, or towel for large cube samples. The box (normally 10 inches cube ID) is then centered over the sample and seated. The box is made of very thin tin plate. Loose soil may be lightly tapped around the out side of the bottom of the box to prevent leakage. Then the soil surrounded by the tin plate is slid into the glass microcosm reactor. Uncontaminated forest soil was recommended for the soil used in these soil microcosm reactor studies. In order to compact the soil cube, some water is sprayed on the soil bed. As soon as the soil bed is transferred to the glass reactor, it is quickly brought into the laboratory, and covered.

Contamination of Soil Bed.

Once the soil had been put into the microcosm reactor, it was contaminated by a mixture of 100 ppm concentration of each of the four nitrate ester compounds. The mixture was sprayed on the top of the soil bed and the water mixture was allowed to infiltrate the soil bed slowly.

Nutrient Supply.

Nutrient solution for the soil bacteria was sprayed twice per day. Nutrient solution for the aerobic microorganism was prepared following the OECD medium [26]. The list of chemicals comprising the medium is shown in Table 3. The nutrient solution contains, 10 mL of solution (a) and 1 mL of each of the following solutions (b) to (f) per liter of water. Total Kjeldahl nitrogen and phosphorus content in the effluent from microcosm reactor is measured on a weekly basis. Depleted components of nutrient solutions are replaced into the nutrient tank at appropriate time intervals.

Operation and Maintenance of the Microcosm Reactor

(1) All the components of the microcosm reactor system are tested and connected before its operation. The entire microcosm system schematic diagram is illustrated in Figure 7. The nutrient tank is pressurized by hand pumping. The pressurized nutrient solution is pushed through the solenoid valve, the manifold, and finally to the sprayer nozzle. Automatic timer is set to open the solenoid valve for 30 seconds and it opens twice a day to provide the nutrient solution. Air gas tank is connected to the inlet on the left side of the microcosm reactor, and outlet on the right side of the reactor to the potassium hydroxide trapping bottle. 0.5 M of KOH solution is prepared for the carbon dioxide absorption to be used to measure biodegradation activity in soil microcosm reactors. The air from gas tank is adjusted to flow at 20 psi. This air supplies the oxygen to the soil bacteria as well as purges out the carbon dioxide from the head space to the KOH solution. The carbon dioxide concentration in the KOH solution is then measured by titrating with hydrochloric acid using phenolphthalein indicator.

(2) Four holes at the bottom of the soil microcosm reactor drain the infiltrate from the soil bed, which flows to the recycle receiving tank. When the recycle tank receives some amount of infiltrate, it is then pressurized in the same manner as the nutrient. The recycle effluent is then transferred to the nutrient tank in the refrigerator through the 2 μ m pore filter. The nutrient component in the recycle effluent is measured weekly, and supplements are added to maintain the constant nutrient requirement. Additionally, the reactor effluent is analyzed for the residual toxic substrates to assess the degree of biodegradation of these compounds in the soil microcosm reactor.

Each microcosm reactor represents a controlled site, which eventually selects out the acclimated indigenous microbial population in the soil for the contaminating organics. Samples of soil are then taken from the microcosm reactors and used as source of acclimated microbial inoculum for measuring (1) oxygen uptake respirometrically; (2) carbon dioxide generation kinetics in shaker flask reactors, and (3) for studies with other soil reactor systems. The microcosm reactor units are also being used directly to evaluate the biodegradability of the pollutant organics and to measure the rate of their biodegradation in this intact, undisturbed soil bed.

Figure 8 shows the cumulative carbon dioxide generation as a function of time. The carbon dioxide evolution by the control microcosm reactor (unspiked with the chemicals) is also shown for comparison. The difference in carbon dioxide evolution provides an indication that the soil microbiota has been acclimatized to the nitrate ester compounds, added to the soil, after a period of 42 days. After the acclimation period of 42 days the cumulative carbon dioxide evolution rate in the contaminated reactor matched the cumulative carbon dioxide evolution rate from the soil control microcosm reactor.

RESPIROMETRIC ANALYSIS OF SOIL SLURRY REACTORS

Respirometric technologies for evaluating and testing biodegradation of organic compounds have gained prominence as reliable methods for quantitating the fate of these compounds in aqueous environments. Electrolytic respirometry approach for quantitating biodegradation and for determining biodegradation kinetics of organic pollutants in aqueous media has only recently been established as a popular procedure through the studies [27,28,29,30,31]. The respirometrically generated biokinetic data have subsequently been used to develop a linear and non-linear group contribution, structure-activity predictive biodegradation model for organics in aqueous systems (28,29).

Extension of the respirometric biodegradation /biokinetics determination protocol to predict the effect of soil on biodegradability of organic pollutants seems to be very desirable. By conducting parallel respirometric biodegradation tests in presence and absence of a soil matrix, it should be possible to determine the biodegradability and biodegradation kinetics of these compounds in soil-slurry systems and in undisturbed soil layers in specially built respirometric reactor systems, as well as deduce the adsorptive properties of an organic pollutant on soil, without the need of extensive chemical analysis. The resulting information could then be used in combination with groundwater and contaminant transport models to assess the potential efficacy of bioremediation of a particular site. Models depicting the combined effects of sorption and biodegradation can be used in simulation studies to investigate how sorptive properties would affect the course of oxygen uptake in batch reactors containing various quantities of soil in a liquid medium or containing undisturbed layers of soil. Sorptive properties of organic compounds on soil can be deduced for oxygen consumption experiments in the presence and absence of soil and these properties can then be compared to experimentally determined adsorption characteristics.

Several research studies in the soil bioremediation/ treatability area using respirometry for development of biodegradation data for organic pollutants in soil have recently been reported and two of those may be cited here. Long-term respirometric BOD analysis was applied to bench-scale studies to continuously monitor bacterial respiration during growth in mixed organic wastes from contaminated water and soil in order to assess the potential for stimulating biodegradation of these wastes [32]. This information was used to make an initial determination regarding the need to further explore bioremediation as a potential remedial action technology using on-site, pilot-scale testing. A treatability study used electrolytic respirometers and biometers to determine the biodegradation potential of crude oil petroleum-based wastes (drilling mud, tary material and heavy hydrocarbons) as contaminants of a polluted soil site area [33]. The treatability data provided biotreatment efficiencies of the petroleum wastes and were used to ascertain the bioremediation clean up time.

Studies were conducted with soil slurry reactors, wherein the oxygen uptake was monitored respirometrically. The extent of biodegradation and the Monod kinetic parameters for variety of the organic pollutant compounds by soil microbiota were determined from oxygen uptake data. Various

concentrations of soil (2, 5, 10 %) and compound (50, 100, and 150 mg/L) were mixed with a synthetic medium consisting of inorganic salts, trace elements and either a vitamin solution or solution of yeast extract and stirred in the respirometric reactor flasks. The flasks were connected to the oxygen generation flask and pressure indicator cells of a 12 unit Voith Sapromat B-12, electrolytic respirometer, and the oxygen uptake (consumption) data were generated as oxygen uptake velocity curves.

A detailed description of the Voith Sapromet B-12 electrolytic respirometer (Voith Inc., Heidenheim, Germany) was presented elsewhere [29], and it includes information on the accessory supportive equipment as well as provides an overview of the mechanism of electrolytic oxygen generation and the plotting of the measured oxygen uptake values.

Experimental Approach

The nutrient solution used in these was an OECD synthetic medium [26] as shown in Table 3, consisting of measured amounts for liter of deionized distilled water of (1) mineral salts solution containing KH_2PO_4 , K_2HPO_4 , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, (2) trace salts solution containing $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, H_3BO_3 , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, (3) vitamin solution containing biotin, nicotinic acid, thiamine, p-aminobenzoic acid, pantothenic acid, cyanocobalamine and folic acid and (4) yeast extract solution as a substitute for vitamin solution. The soil served as a source of inoculum. The concentration of forest soil in the reactor flask varied from 5 to 15 % by weight, using dry weight of soil as the basis. The total volume of the slurry in the flask was 250 ml.

The test and control compound concentrations in the media were 100 mg/L. Aniline was used as the biodegradable reference compound at a concentration of 100 mg/l. The typical experimental system consisted of duplicate flasks for the reference substance, aniline, and the test compounds, a single flask for the physical/chemical test (compound control), a single flask for toxicity control. The contents of the reaction vessels were preliminarily stirred for an hour to ensure endogenous respiration state at the initiation of oxygen uptake measurements. Then the test compounds and aniline were added to it. The reaction vessels were then incubated at 25°C in the dark (enclosed in the temperature controlled waterbath) and stirred continuously throughout the run. The microbiota of the soil samples used as an inoculum were not pre-acclimated to the substrates. The incubation period of the experimental run was 28 to 50 days. A more comprehensive description of the procedural steps of the respirometric tests is presented elsewhere [29].

Results and Discussion

Figure 9 shows the cumulative oxygen uptake by Nitroglycerine for concentrations of 100 mg/L and 200 mg/L. The soil concentration selected for the study was 10%, since 5% produced low oxygen uptakes and 15% tended to settle down at the bottom of the flasks. At 100 mg/L concentration, the cumulative oxygen uptake plateaued at about 27 mg/L in 20 days. At 200 mg/L the oxygen uptake was significantly lower indicating that the compound was toxic to the microbiota at this concentration. The control contained 10% soil mixed with nutrients dissolved in distilled deionized water with no chemical

present. Natural soil respiration produced very low oxygen uptake rates.

To demonstrate the toxicity effects, the compound, nitroglycerine, was mixed with 100 mg/L of aniline and respirometric studies were conducted with this mixture. As shown in Figure 10, aniline degraded initially producing an oxygen uptake of about 260 mg/L followed by the degradation of Nitroglycerine. This indicated that the presence of nitroglycerine did not inhibit the degradation of aniline, an easily degradable compound. For a concentration of 200 mg/L, aniline degradation was severely inhibited.

Figure 11 shows the oxygen uptake for PGDN at two concentrations of 100 mg/L and 200 mg/L. Even with acclimated soil from the microcosm reactor, there was an initial lag period of about 12 days. However, 100 mg/L of PGDN degraded in about 24 days giving a reasonable cumulative oxygen uptake curve. Oxygen uptake curve for a concentration of 200 mg/L was severely inhibited indicating toxicity effects. This was also seen from the PGDN with aniline data shown in Figure 12.

Figure 13 shows the oxygen uptake curve for TEGDN at two concentrations of 100 mg/L and 200 mg/L. Again toxicity effects were found to occur at 200 mg/L concentration. There was an initial lag period of about 15 days before oxygen uptake occurred even for 100 mg/L concentration. Figure 14 shows the results with a mixture of aniline and TEGDN, showing inhibition of aniline degradation due to the compound at a concentration of 200 mg/L.

Figures 15 and 16 show the oxygen uptake and aniline degradation data for TMETN. Oxygen uptake at 100 mg/L concentration had an initial lag period of 24 days. There was no difference between the oxygen uptake at 200 mg/L and the control and hence the figure for 200 mg/L concentration was deleted.

These results indicated that soil microbiota could be acclimatized at concentrations of 100 mg/L for all four compounds. The microcosm reactor was spiked with 100 mg/L concentration of all four compounds and the results showed that all four compounds degraded aerobically at 100 mg/L concentration. The possibility of obtaining acclimated microbiota at higher concentrations was explored but the experiments were unsuccessful. Even when the microcosm reactor was spiked with higher concentrations, toxicity effects were found to occur at concentrations exceeding 100 mg/L. Furthermore, for concentrations exceeding 100 mg/L, the pH in the reactor flasks in the respirometer began to decrease due to acid formation, and in order to buffer the pH change, larger reactor flasks were necessary. Since we did not have larger respirometer flasks, experiments at concentrations above 100 mg/L were not conducted to determine the highest concentration before the onset of toxicity effects to the soil microbiota.

BIODEGRADATION KINETICS AND KINETIC MODELS FOR BIODEGRADATION IN SOIL SYSTEMS

Several models, either empirical or theoretical, have been proposed to represent the kinetics of biodegradation in soil. These include first order, Michaelis-Menten, zero-order, half-order, and mixed-order models.

Application of existing models for biodegradation is questionable due to the following reasons: 1) diffusional barriers in soil macro and micro pores have been neglected; 2) many organics sorbed to clays and humic constituents, and degradation of sorbed compounds is different than compounds in solution; 3) effect of other chemicals that can be metabolized, such as organic matter in soil; 4) diffusion of nutrients or oxygen may be limiting; 5) protozoa parasitizing the biodegrading population may govern the growth of the population; 6) certain compounds may be dissolving in the water before degradation and certain compounds, with low solubility, may not; 7) the active cells may be sorbed on the clay and humic materials, and kinetics and growth of sorbed microbes is different than suspended or aggregate cultures; and 8) all kinetic models ignore the acclimation period.

Most biodegradation kinetic models have neglected sorption of the contaminant on soil particles, which has been shown to be important in contaminant transport and kinetically is manifested as a two-phase process, with an initial fast stage (< 1 hour) followed by a slower long phase (days), controlled by diffusion to internal adsorption sites [34].

Soil consists of various size pores, with about 50% of the total pore volume consisting of pores with radii < 1 μm . Most soil bacteria range in size from 0.5 to 0.8 μm , and hence a significant portion of the soil may be inaccessible to most bacteria.

The role of soil aggregates and their characteristics on bioremediation in soil has received very little attention. The contaminated aggregates bioremediation (CAB) model has been developed and simulated to analyze the bioremediation of soil and water in the aggregates [35]. The model equations consist of a system of three non-linear partial differential equations. Dimensional analysis of these equations has been performed. Sensitivity analysis conducted by numerically solving them, has demonstrated the effects of aggregate radius, partition coefficient, and initial contaminant concentration on the time and mechanism of remediation. The rates of diffusion of substrate and oxygen, and the biodegradation rates were found to be the controlling mechanisms for remediation in the aggregates.

A two-dimensional finite difference model to study the simultaneous movement of dense nonaqueous phase liquid and water in heterogeneous porous media was developed. This model was applied for the simulation of ground water contamination problems involving the advance of immiscible liquids into previously uncontaminated ground water system. The numerical model was verified against an exact analytical solution which incorporated fully the effects of both relative permeability and capillary pressure. The model was validated through comparison to a parallel plate laboratory experiment involving the infiltration of tetrachloromethylene into a heterogeneous sand pack. A numerical model incorporating

axial dispersion, first-order reversible adsorption, and several forms of a biological reaction term was developed to simulate exponential responses of microbially active soil columns. The use of a Monod type biological reaction term adequately represented the behavior throughout bioreactor operation. Organic solutes were divided into two fractions, utilizable and non-utilizable as microbial substrate on a percentage basis. Calibration and verification of the model was accomplished by utilizing data for several experiments.

An effort was made to evaluate mathematical model of the transport of biologically reacting solutes in saturated soils and aquifers. A mathematical model was derived that involves no unwarranted assumption about the distribution of the microorganisms in the pore space. The governing equations are those using the concept of microcolony and are identical to those that would result from adopting a simple form of biofilm model to describe bacterial growth in the pore space.

Biodegradation Kinetics

Scow, et al. [36] in their review of studies on the kinetics of biodegradation in soil, discussed biodegradation processes linked to growth, biodegradation by nongrowing organisms; zero-order kinetics; first-order kinetics and Monod kinetics and their usefulness to biodegradation in soil. Their review also provided information on biodegradation kinetics in complex environments in soil; the effects of diffusion and adsorption on biodegradation; the two-compartment kinetics applicable to availability and unavailability of the chemical for biodegradation in soil compartments; the metabolism of one substrate during growth of another; the three-half order kinetics and the kinetics of fungal processes. They concluded that models formulated to date rely on concepts derived from studies of single populations or single enzymes. Given the mixture of chemicals, the complexity of soil environments and the variety of microbiota in soil that may bring about biodegradation, it is unlikely that a single model or equation would be useful for the description of rates of biodegradation of all organic substrates in all soil environments.

The success of bioremediation processes lies in : (1) degrading the organic contaminants and (2) reducing both the toxicity and the migration potential of the hazardous constituents in soil. Laboratory studies was conducted using phenolic compounds to characterize overall chemical degradation and toxicity reduction in a contaminated soil for a solid phase bioremediation process. The results indicated that (1) first-order kinetics satisfactorily fit the loss of phenolic compounds and the decrease of toxicity that occurred; (2) contaminant loss in the water soluble fraction was faster than loss of the some chemicals in soil; (3) the toxicity of water soluble fraction decreased as the soil chemical and water soluble fraction chemical concentration decreased; and (4) no enhanced mobilization of the applied chemicals resulted as the degradation and detoxification occurred. Results suggest that by-products of the degradation and chemical loss mechanisms do not increase the toxicity of water soluble fraction from such processes.

The kinetics of ultimate biodegradation (mineralization to CO_2) of linear alkylbenzene sulfonate (LAS) was studied in sludge-amended agricultural soils for a series of pure chain length LAS homologs.

Degradation rates were measured by following the production of $^{14}\text{CO}_2$ from uniformly ^{14}C -ring-labeled material. On the basis of the results of these studies and those of other investigators, it can be concluded that soil environments exposed to LAS in sewage sludges contain microbial concentration which can actively metabolize the material.

The kinetics of phenolic compounds removal in soil were studied [29] and zero order and first order kinetic models were evaluated to determine their adequacy to describe the removal of seventeen phenolic compounds in soil. Both models were shown to describe the removal of these phenols from soil with high correlation coefficients. A kinetic model which incorporates microbial growth may be desirable to describe the removal of certain compounds when removal is associated with growth. Future studies should thus determine the importance of microbial growth in the removal kinetics of hazardous waste constituents in soil systems.

Kinetic Analysis of Soil Slurry Oxygen Uptake Data

The oxygen uptake data were analyzed by computer simulation techniques and curve fitting methods, using the Monod equation combined with a nonlinear adsorption isotherm, to determine the soil adsorption parameter, and the Monod equation biokinetic parameters. The experimental data were analyzed using a mathematical model (equations given below) which include the effect of chemical adsorption / desorption on the soil particles and the adsorption / desorption of the microorganisms from the soil.

$$X_s = K_b X_w \quad (1)$$

$$S_s = K_d S_w^{1/n} \quad (2)$$

$$O_2 = (S_{t0} - S_t) - (X_t - X_{t0}) - (S_p - S_{p0}) \quad (3)$$

$$dX_t/dt = (\mu_w X_w S_w)/(K_w + S_w) + \mu_{sw} X_s S_s/(K_{sw} + S_s) - (b K_w X_t)/(K_w + S_t) \quad (4)$$

$$dS_t/dt = -(1/y)(\mu_w X_w S_w)/(K_w + S_w) - (1/y)(\mu_{sw} X_s S_s/(K_{sw} + S_s))(w/v) \quad (5)$$

$$dS_p/dt = -y_p(dS_t/dt) \quad (6)$$

where subscripts t, s, w and p represent the total, soil, water and degradation products respectively. S is the concentration of compound, X is the concentration of biomass, and S_p is the concentration of the degradation products. μ_w , K_w , y and y_p are the Monod equation maximum specific growth rate parameter, Michaelis constant, biomass yield and product yield coefficient, respectively for the water phase. μ_{sw} , K_{sw} are the Monod equation maximum specific growth rate parameter and Michaelis constant, respectively for the soil phase. b is the biomass decay coefficient, K_d is the soil adsorption

isotherm parameter, $(1/n)$ is the soil adsorption intensity coefficient, and K_b is the biomass adsorption parameter. O_2 is the cumulative oxygen uptake. w is the weight of soil in the slurry reactor and v is the total volume of water in the reactor.

The experimental values of the oxygen uptake were matched with the theoretically calculated values and the best fit for the parameters was obtained using an adaptive random search method. Table 4 shows the biokinetic parameters determined from the oxygen uptake curves. Clearly nitroglycerine (NG) has the highest biodegradation rate compared to the other three compounds studied. Only oxygen uptake data for 100 mg/L concentration were analyzed to obtain the biokinetic parameters.

STUDIES ON CARBON DIOXIDE EVOLUTION IN SOIL SLURRY REACTORS

Microbial respiration (uptake of oxygen and/or release of carbon dioxide) in soil, which includes the exchanges of gases that result from both aerobic and anaerobic metabolism, has been used as a very reliable tool for assessing the fate (biodegradation/mineralization) of the organic pollutants in soil. Anderson [37] provided an extensive review of the methodology and apparatus for measurement of oxygen consumption and carbon dioxide evolution rates in laboratory and field application. He reported on the procedures for measurement of $^{14}CO_2$ evolution rates and the special apparatus for use in studies with radiolabeled compounds; and discussed the significance of the O_2 consumption and CO_2 evolution data for quantitation of biodegradation of organic pollutants in soil microenvironments.

Studies have been reported in the literature which relate to the significance of measurement of CO_2 evolution rates in soil matrices for the evolution of the biodegradation/mineralization of the organic pollutant compounds in soil. Gledhill [38] developed a screening test for assessment of ultimate biodegradability of linear alkylbenzene sulfonates by soil microorganisms which incorporates the use of specially equipped shaker flasks for assaying CO_2 evolution. Birch and Fletcher [39] have applied dissolved inorganic carbon measurements to the study of aerobic biodegradability and developed an improved form of CO_2 production test for assessing ultimate biodegradation of volatile, and sparingly soluble compounds by soil and sewage microbiota under aerobic conditions.

Comparison of methods for microbial activity measurements in soil were made by Bauer, et al. [40] and CO_2 determination method was judged to be fairly sensitive in comparison to the enzyme activity analysis (TTC and INT dehydrogenase activity, DMSO reduction) method. Studies of Edwards and Sollins [41] employed an infrared gas analyzer (IRGA) to obtain continuous measurements of CO_2 evolution for partitioned forest floor components, and the respiration measurements of CO_2 evolution provided reliable data on biodegradation of certain carbohydrates. An environmental assessment of aerobic biodegradation in soil by FDA [42] demonstrates the significances of CO_2 evolution test data for the determination of the extent and rate of biodegradation in addition to the measurement of the removal of the compound itself, uptake of O_2 and the increase in microbial biomass. Studies of Reiners [43] on

CO₂ evolution for the floor of Minnesota forests have reported on the reliability of CO₂ generation data as a measurement of bioactivity in forest soil.

Carbon dioxide generation rates were measured in shaker-flask soil slurry systems and in the electrolytic respirometry soil-slurry reactors in order to assess the rate and extent of biodegradation/mineralization of alkyl phenols in soil slurry. The CO₂ generation rate measurement serves as an additional tool for quantitating the biodegradation of these organics to the cumulative respirometric oxygen uptake data from which the biokinetics of biodegradation data were derived. The CO₂ generation rate measurement experiments were performed using both the shaker flask and respirometric reactors to determine the compatibility and reproducibility between the data on CO₂ production in both systems.

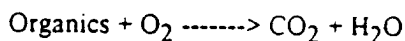
Soda lime was used initially as the absorbent for CO₂ evolution studies in the shaker flask and in respirometric reactors since soda lime served ordinarily as the absorbent of choice in the oxygen uptake respirometric studies for determination of biodegradability and biodegradation kinetic parameters of the priority pollutant organics. Subsequently soda-lime was replaced by KOH as the absorbent in the CO₂ evolution measurement studies, because of the observed slower rate of release of CO₂ from soda-lime during the analysis of CO₂.

Four methods were used for CO₂ measurement in the shaker flask soil slurry systems and in respirometric reactors: (1) use KOH to trap CO₂ released from soda lime, and then titrate KOH using standard HCl before and after CO₂ absorption; (2) use Ba(OH)₂ to trap CO₂ released from soda lime, and then titrate Ba(OH)₂ using standard HCl before and after CO₂ absorption; (3) use KOH to trap CO₂ released from soda lime, precipitate the KHCO₃ and K₂CO₃ formed, using BaCl₂ to get BaCO₃ which is filtered, dried and weighed; (4) use KOH to trap CO₂, measure pH of KOH solution before and after CO₂ absorption, calculate CO₂ generated according to the changes of pH values using the developed computer program [20]. The pH method was shown to be most accurate and provided the most reproducible data. Shaker flask slurry systems data on CO₂ production were generated for the same alkyl phenols used in the respirometric soil slurry reactor studies.

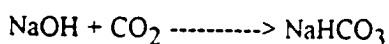
The chain of events covering the generation of CO₂ during the metabolic activity of soil microbiota on phenolic compounds in soil slurry systems, the absorption and analysis of absorbed CO₂ can be divided into three phases.

Phase 1 - Biodegradation, CO₂ Generation and Absorption

The biodegradation in soil and CO₂ absorption is expressed as follows:



The CO₂ absorption using soda-lime is expressed as follows:

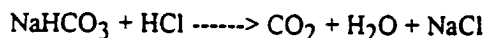


Phase 2 - Release of Absorbed CO₂ from soda lime and Absorption of CO₂ by either KOH or Barium Hydroxide Solution

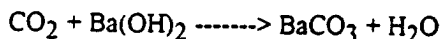
The CO₂ absorbed soda lime was transferred into a reaction flask with three necks to be processed for

analysis. 50 mL of 3.0 N HCl solution is added to release the CO₂ from soda-lime. The generated CO₂ is flushed by a constant flow of nitrogen gas through either 80 mL of 0.3 N KOH or barium hydroxide solution.

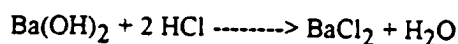
The CO₂ release from soda lime by HCl:



The CO₂ absorption by barium hydroxide:



Phase 3 - Determination of CO₂ Evolution. In the above process, carbon dioxide was trapped as BaCO₃. To quantify the amount of CO₂ a titration method was applied. CO₂ trapped as BaCO₃ is quantified by titration of the free (unused) barium hydroxide with 0.2 N HCl to the phenolphthalein end point. The reaction of free barium hydroxide with HCl is expressed as follows:



The final carbon dioxide concentration is determined using the equation,

$$\text{CO}_2, \text{mg/L} = (V_1 - V_2) \times (N_{\text{HCl}}) \times (44/2) \times (1000/125)$$

where, V_1 = volume of HCl titrated with fresh Ba(OH)₂, mL

V_2 = volume of HCl titrated with free Ba(OH)₂ after absorption

N_{HCl} = normality of HCl solution

Carbon Dioxide Generation Rate Measurement in Shaker Flasks.

Carbon dioxide generation rates were measured in shaker flasks fitted with ground joints to hold cylindrical tubes containing the soda lime absorbent. Varied soil sample concentrations were mixed with OECD nutrients and several phenolic substrate concentrations in a distilled deionized water. The glass columns were filled alternatively with sterile cotton and soda lime; the upper bed of soda lime pellets was used to absorb the atmospheric CO₂ and the lower bed of soda lime pellets was used to absorb CO₂ generated in the flask as a result of microbial metabolic activity. At specified sampling time periods, replicates of shaker flasks and their contents were sacrificed and the inner soda lime layers of the glass columns were analyzed for the amount of CO₂ generated using the three procedures described for soda lime absorbent.

In shaker flask soil slurry systems, incorporating KOH solution as absorbent for the generated CO₂, specially designed flasks with interchangeable KOH traps were incorporated in the study, so that by replacing these traps with fresh KOH solution, continuous CO₂ evolution for the duration of the incubation time can be measured for each shaker flask. The pH method using the computer program was used to calculate the continuous CO₂ generated from each flask in these shaker flask soil slurry systems.

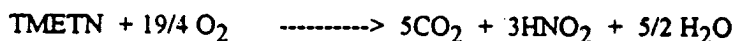
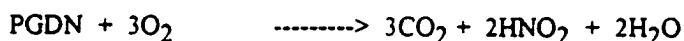
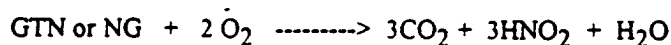
Carbon Dioxide Generation Rate Measurement in Respirometric Reactors.

Carbon dioxide generation rates were measured in the respirometric reactors utilizing both soda

lime and KOH solution as the absorbents of the generated CO_2 . The CO_2 evolution studies were undertaken at the same time that oxygen uptake data were generated in the same respirometric reactors for determination of biokinetics of degradations of the alkyl phenols. In experiments in which soda lime was used as absorbent, at appropriate sampling times during the respirometric runs, coincident with designated times of the respirometric oxygen uptake velocity curve, total soda lime amounts were emptied from the reservoirs in the reactors and subjected to the three procedures described for soda lime absorbent. The soda lime that was analyzed was replaced with fresh soda lime in the reservoirs for subsequent CO_2 evolution measurement. This sampling procedure provided CO_2 evolution data for the entire respirometric oxygen uptake experiment. In the experiments using KOH solution as the absorbent, the pH method using the computer program was used to calculate the continuous CO_2 generated from each respirometric reactor in the respirometric soil slurry systems.

Results and Discussion

The carbon dioxide evolution rates for concentrations of 100 mg/L and 200 mg/L are shown in Figures 17 through 20. The following equations were used to determine the theoretical oxygen uptake requirements for each compound.



The figures clearly show that incomplete mineralization was found to occur at concentrations of 200 mg/L. However, for concentrations of 100 mg/L, all four compounds were found to mineralize completely. It is also clear that the rate of carbon dioxide evolution does not necessarily match the oxygen uptake rates. This was mainly due to carbon dioxide dissolution in the liquid medium followed by eventual desorption as the carbon dioxide concentration decreased in the gas phase.

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Table 1. Characteristics of soil used in this study.

Total Carbon	0.415%
Carbonate	negligible
Organic Carbon	0.415%
Organic Matter	0.80%
Particle size distribution	
< 2 mm	15.65% by weight
2-10 mm	5.00%
10-20 mm	7.75%
20-44 mm	7.90%
44-75 mm	4.55%
75-150 mm	6.50%
150-300 mm	14.30%
300-600 mm	22.10%
600-1180 mm	16.25%

Table 2. Retention Time (min.) for the compounds studied.

Compound	Acetonitrile	DIUF Water	Flow rate (ml/min)	Retention Time
Nitroglycerine	36%	64%	3.0	13.0
PGDN	36%	64%	3.0	7.0
TEGDN	50%	50%	1.0	9.8
TMETN	50%	50%	1.0	15.0

Table 3. OECD Aerobic Medium (1983) [26]

(a)	KH_2PO_4	8.50 g
	K_2HPO_4	21.75 g
	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	33.40 g
	NH_4Cl	2.50 g

Dissolve in and make up to 1000 mL with distilled water. The pH should be 7.2.

(b)	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	22.50 g
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Dissolve in and make up to 1000 mL with distilled water.

(c)	CaCl_2	27.50 g
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Dissolve in and make up to 1000 mL with distilled water.

(d)	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.25 g
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Dissolve in and make up to 1000 mL with distilled water. This solution is freshly prepared immediately before use.

(e)	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	39.9 mg
	H_3BO_3	57.2 mg
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	42.8 mg
	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$	34.7 mg
		= 36.85 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$
	$\text{FeCl}_3 \cdot \text{EDTA}$ (Fe - Chelate)	
		100.0 mg

Dissolve in and make up to 1000 mL with water.

Sterilization of the trace element stock solution at (120°C), 2 atm, 20min.

(f)	Vitamin solution
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Biotin	0.2 mg
Nicotinic acid	2.0 mg
Thiamine	1.0 mg
p-Aminobenzoic acid	1.0 mg
Pantothenic acid	1.0 mg
Pyridoxamine	5.0 mg
Cyanocobalamine	2.0 mg
Folic acid	5.0 mg

Dissolve in and make up to 100 mL with water.

The solution is filtered sterile through 0.2 μm membrane filters. Instead of solution (f) 15 mg of yeast extract may be used per 100 mL of water.

Solutions (e) and (f) may be omitted.

Table 4. Biokinetic Parameters for the Compounds Studied

Kinetic Parameters	NG	PGDN	TEGDN	TMETN
μ_w (1/hr)	0.127	0.082	0.098	0.077
K_w (mg/L)	3.47	10.2	4.96	12.5
μ_{sw} (1/hr)	2.26	3.20	2.06	1.75
K_{sw} (mg/L)	1.15	15.7	4.85	7.90
y (mg/mg)	0.12	0.14	0.12	0.09
y_p (mg/mg)	0.000693	0.000949	0.000960	0.000655
b (1/hr)	0.000438	0.000676	0.000564	0.000818

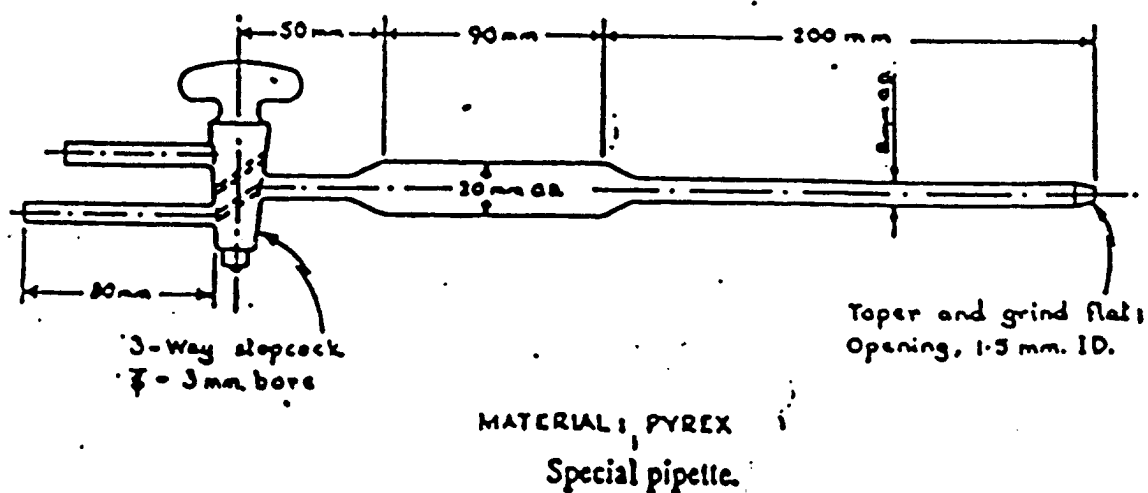
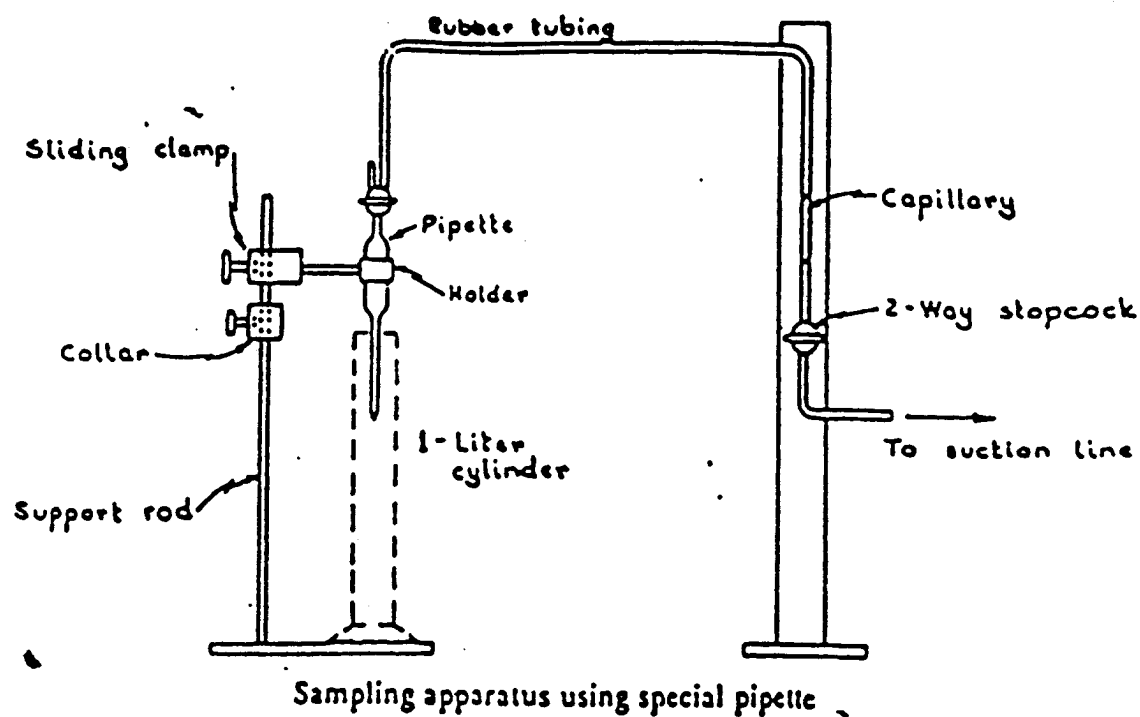


Figure 1. Experimental System for particle size determination.

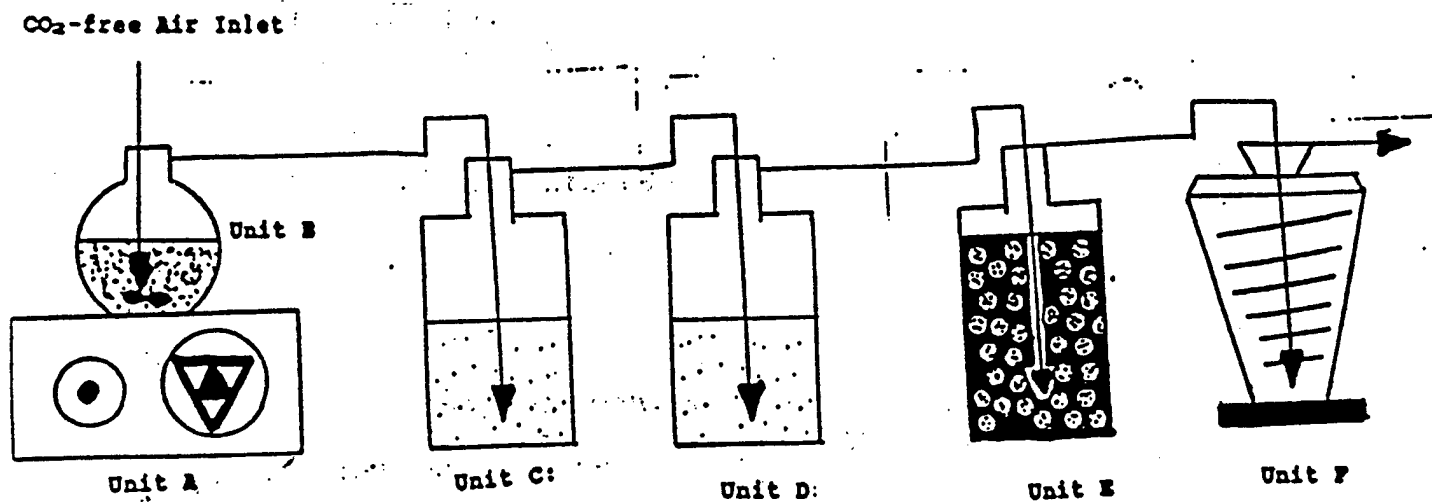


Figure 2. Apparatus for total carbon content determination.

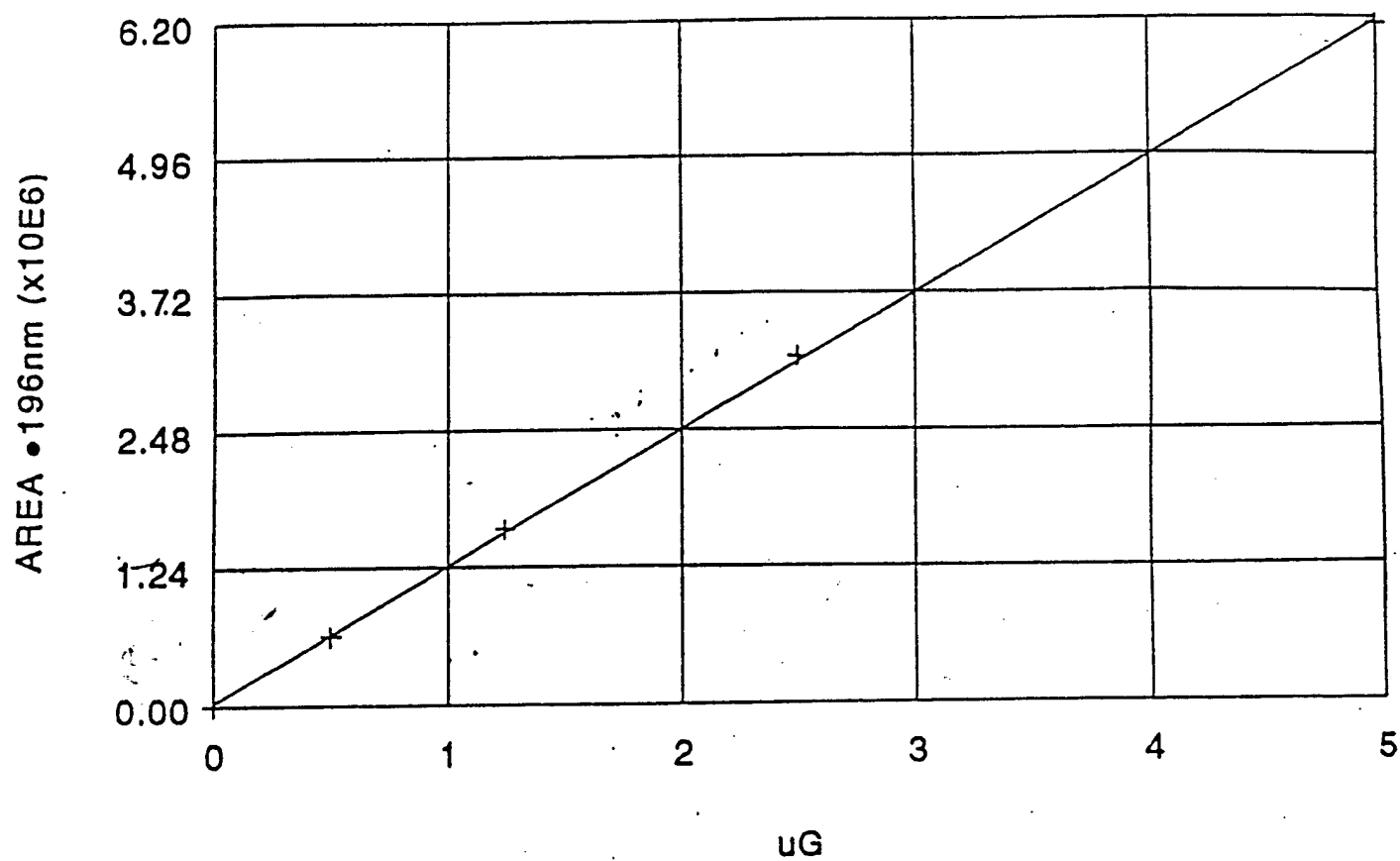


Figure 3. Standard curve for Nitroglycerine (NG) or Glycerol Trinitrate (GTN).

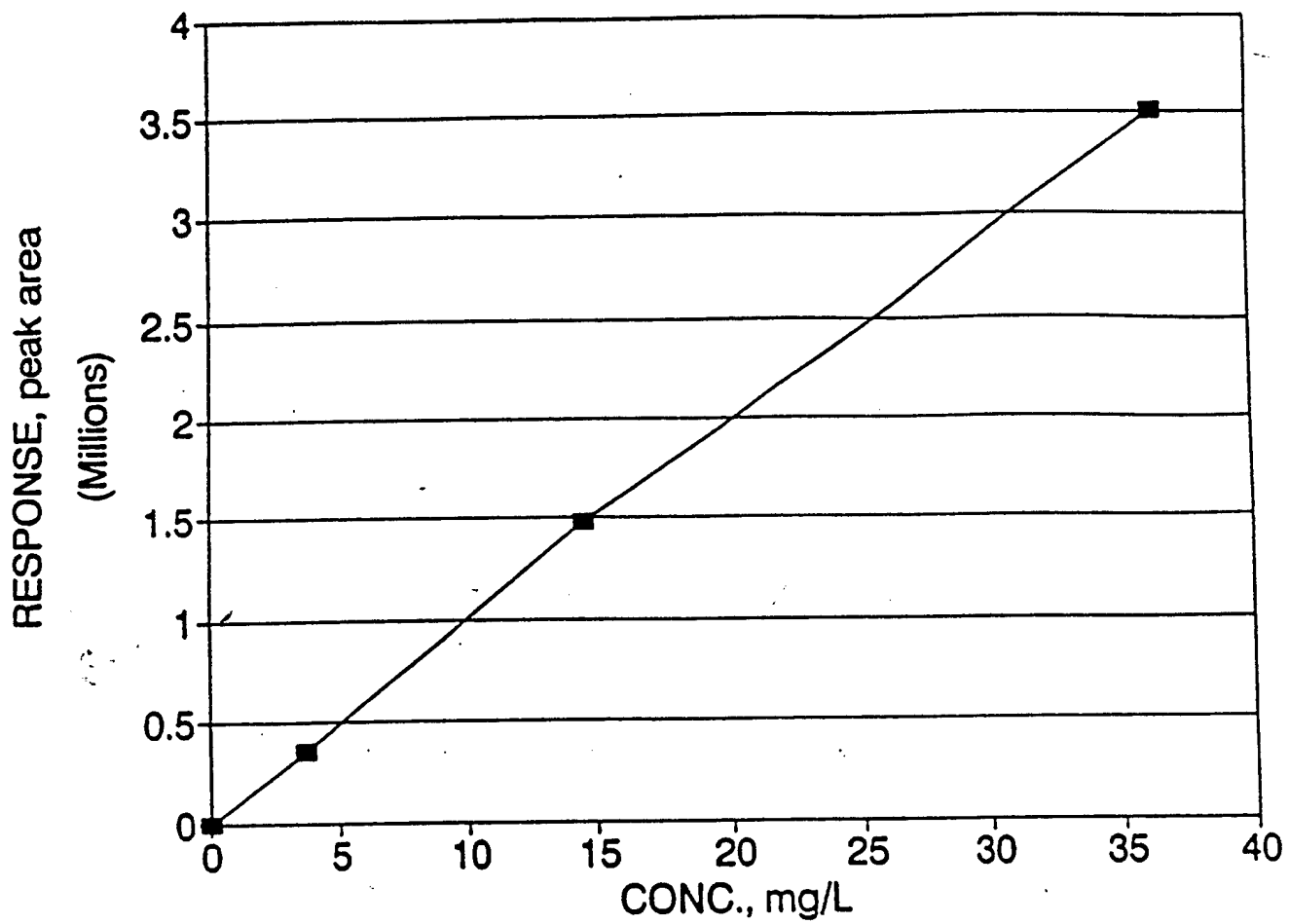


Figure 4. Standard curve for Propylene Glycol dintrate (PGDN).

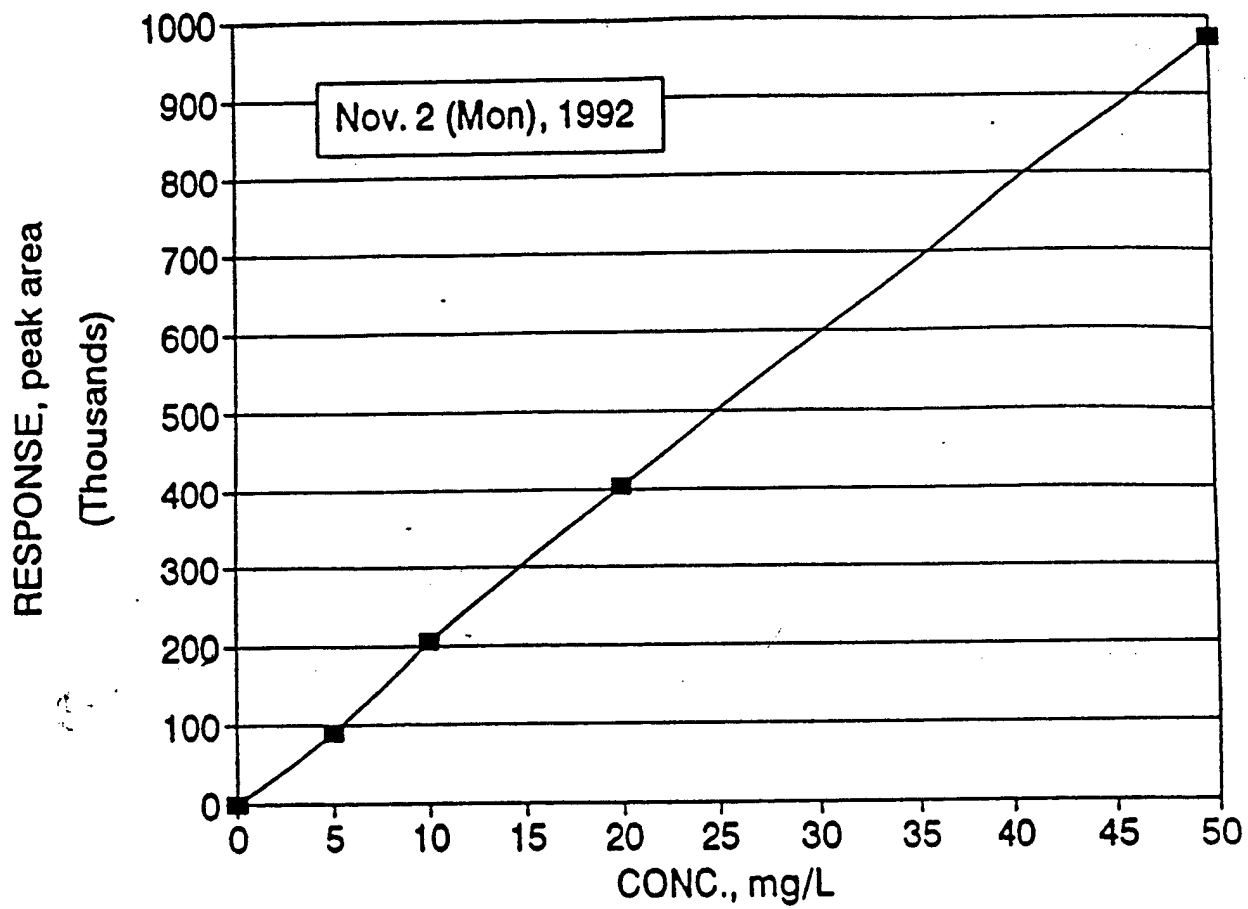


Figure 5. Standard curve for Triethylene glycol dinitrate (TEGDN).

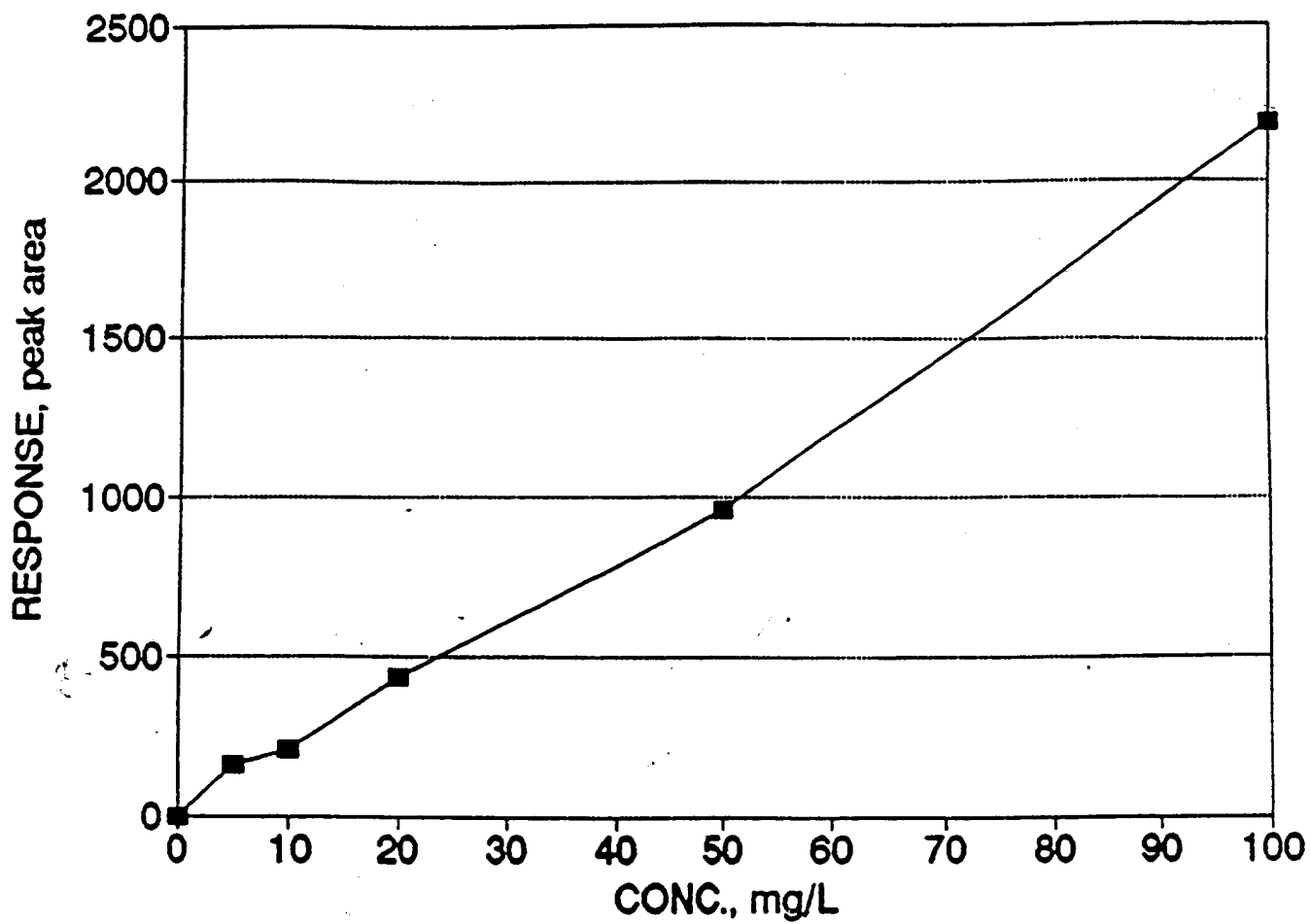


Figure 6. Standard curve for Trimethyloethane trinitrate (TMETN).

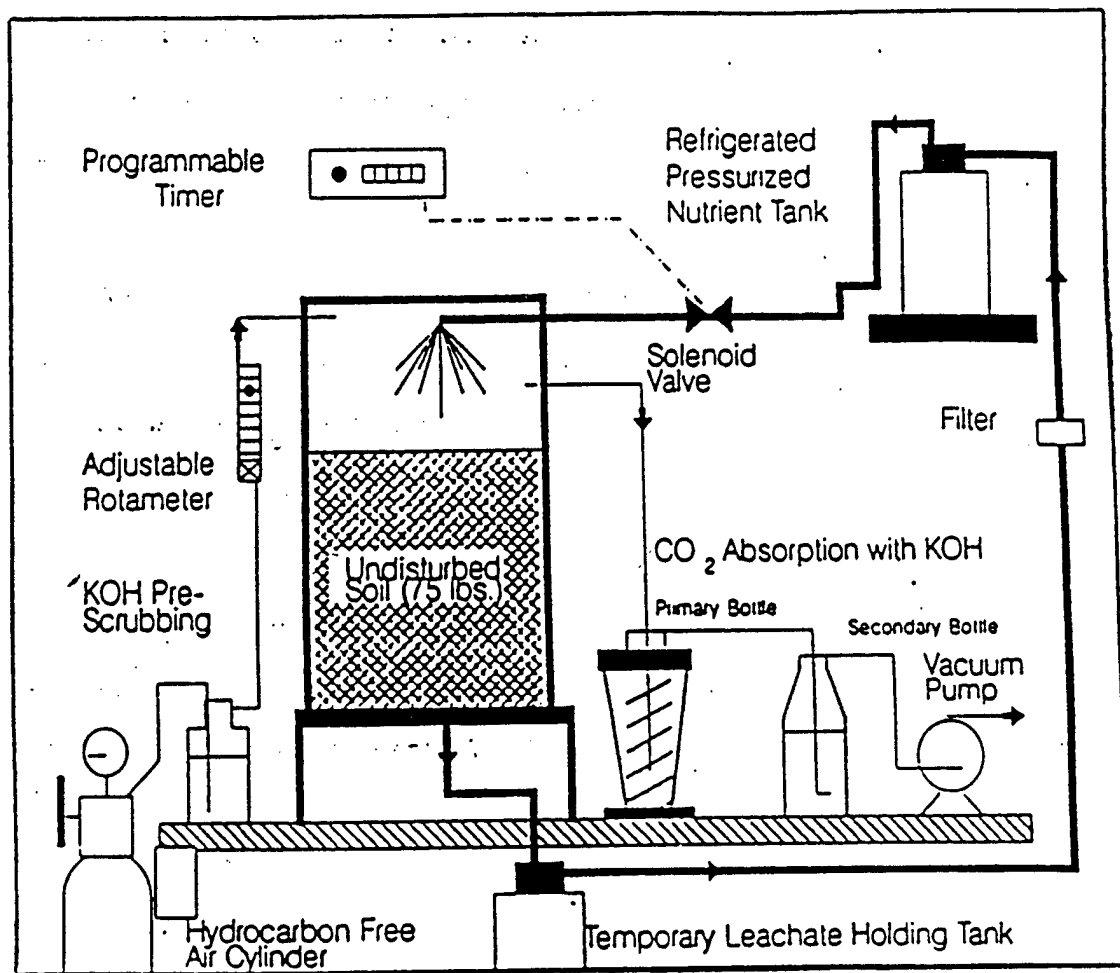


Figure 7. Schematic of the Soil Microcosm Reactor System.

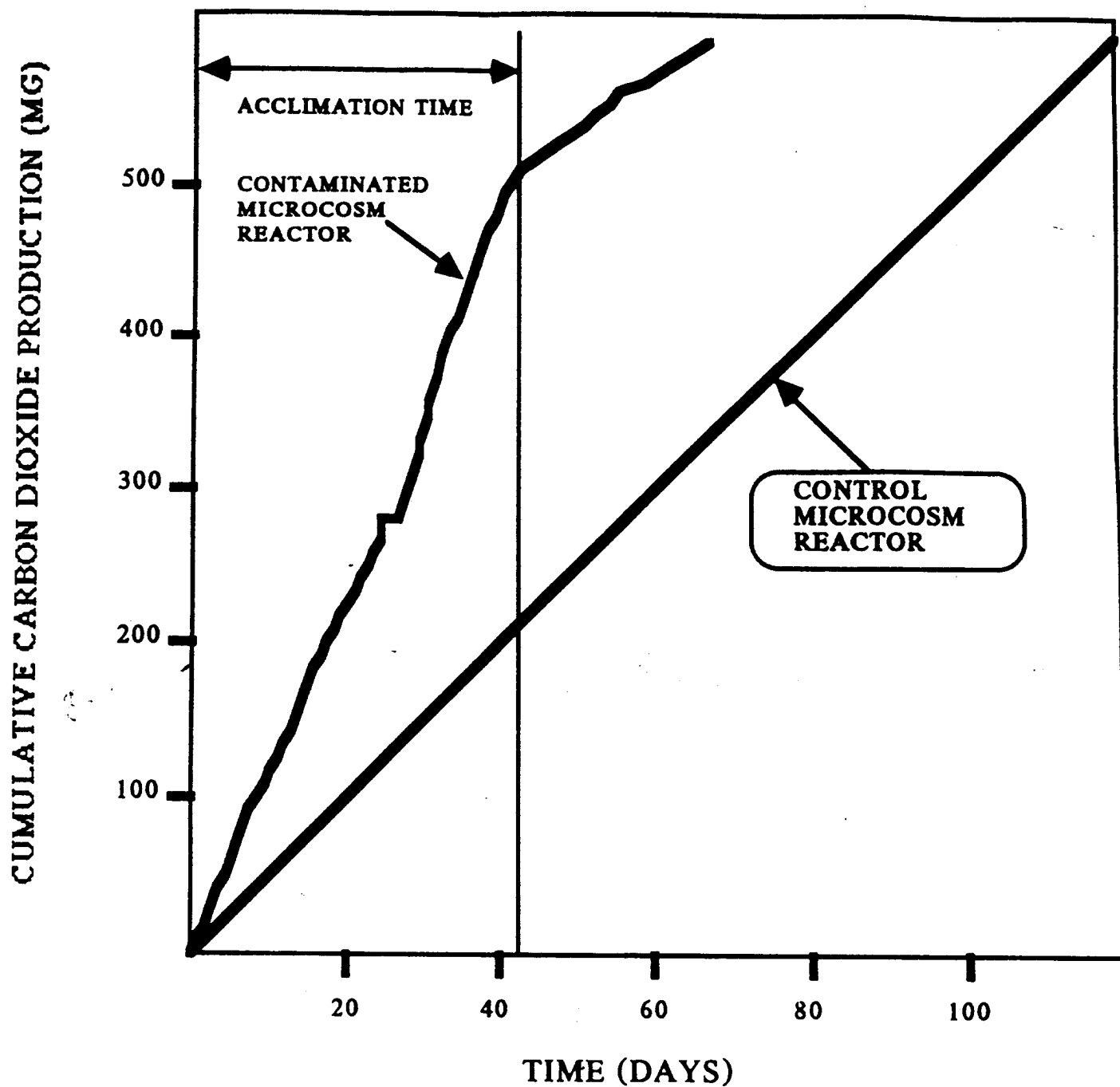


Figure 8. Plot of Cumulative Carbon dioxide Evolution from the contaminated and control Microcosm Reactors.

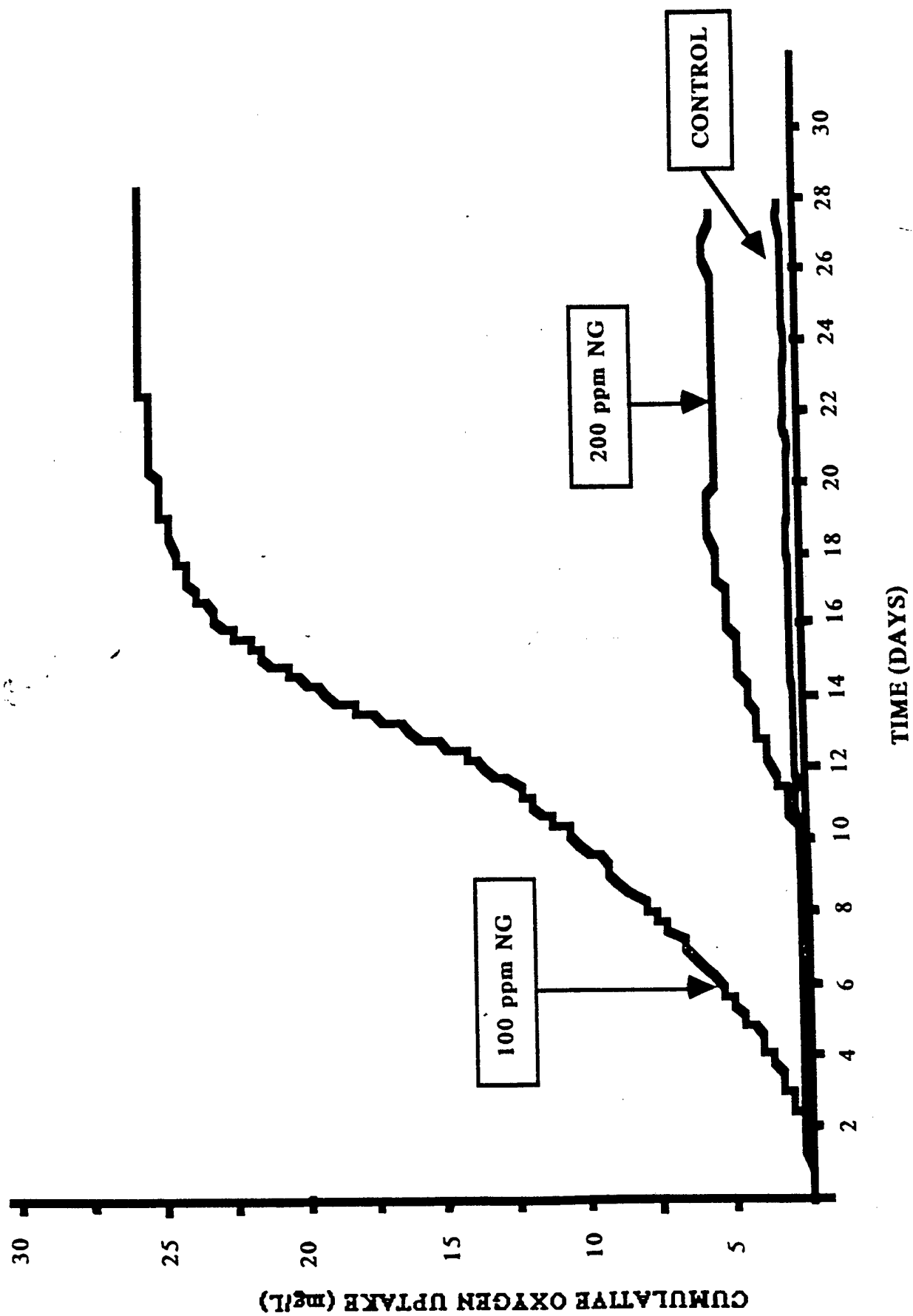


Figure 9. Cumulative Oxygen Uptake for Nitroglycerine (NG) in a soil slurry reactor with 10% soil suspension and initial NG concentrations of 100 mg/L and 200 mg/L.

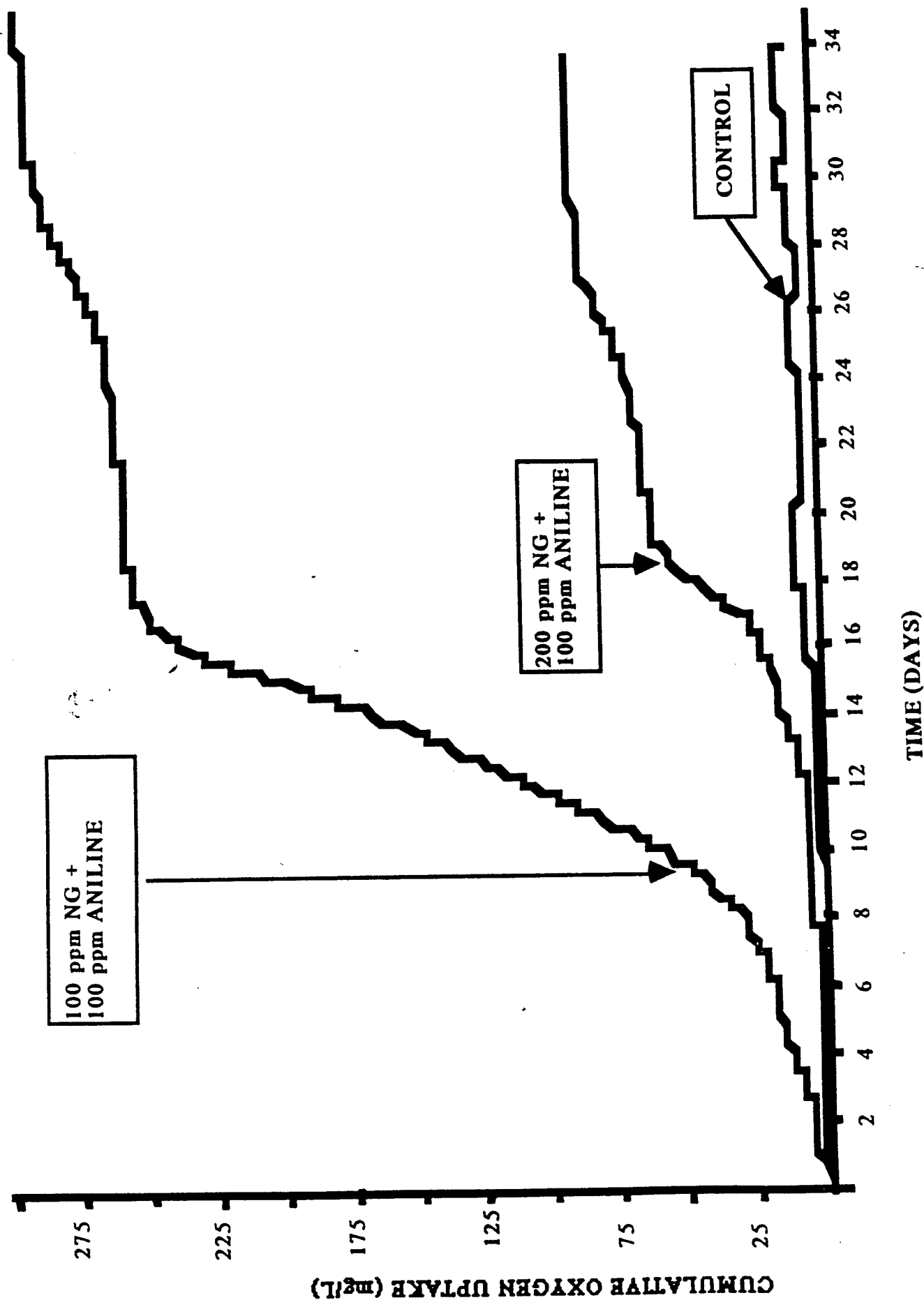


Figure 10. Cumulative Oxygen Uptake for Nitroglycerine (NG) and Aniline in a soil slurry reactor with 10% soil suspension.

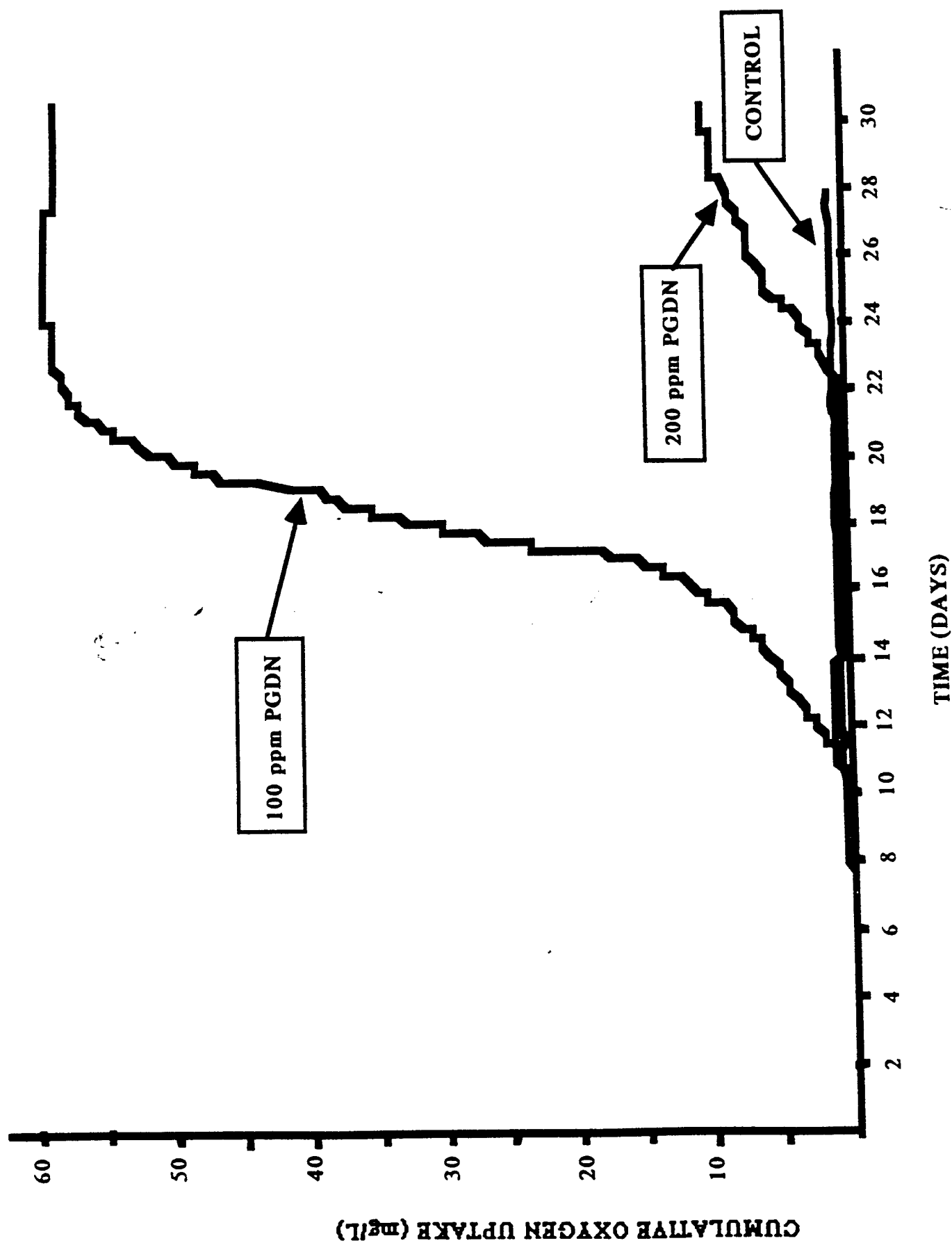


Figure 11. Cumulative Oxygen Uptake for Propylene Glycol dinitrate (PGDN) in a soil slurry reactor with 10% soil suspension and initial PGDN concentrations of 100 mg/L and 200 mg/L.

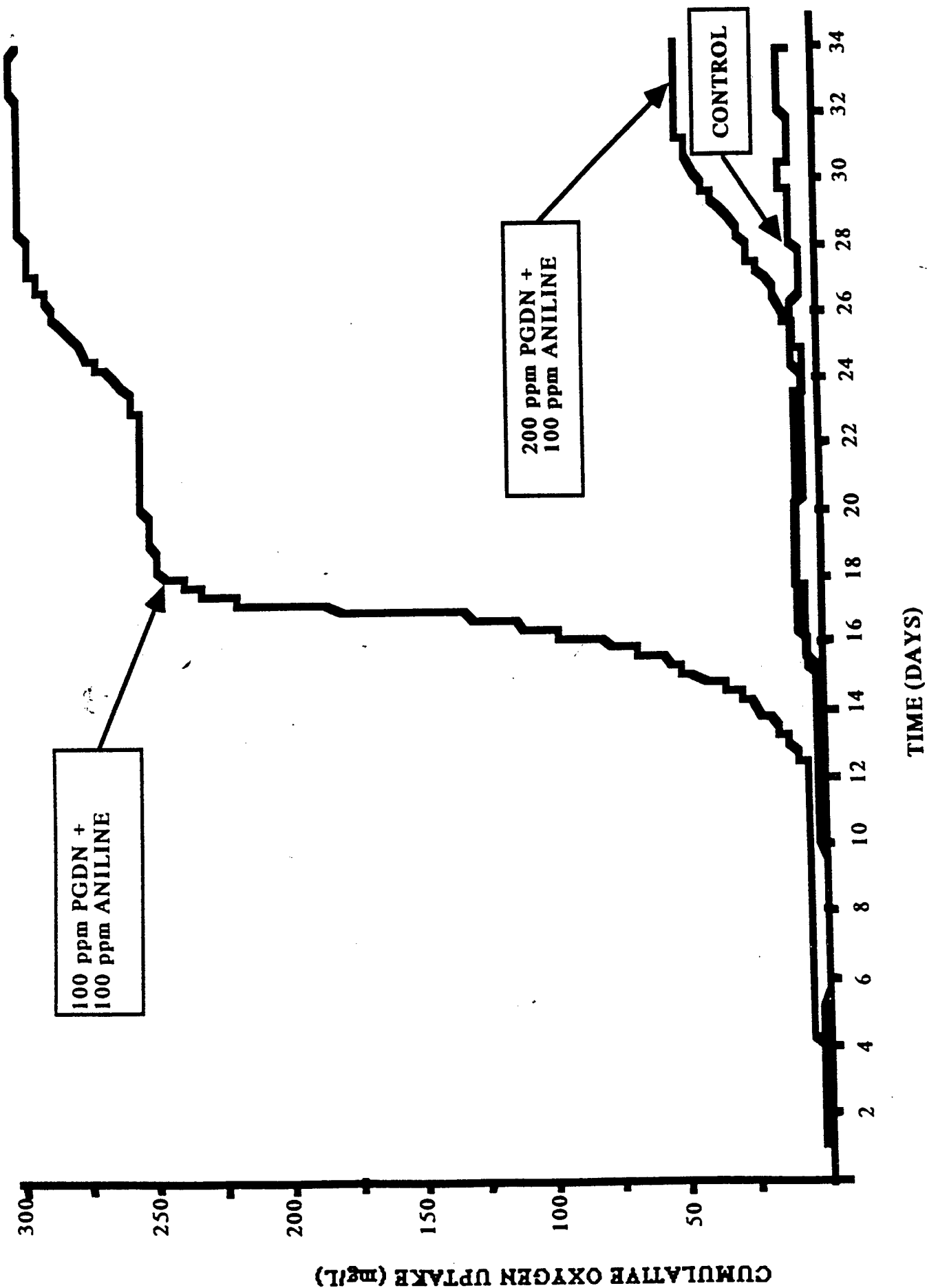


Figure 12. Cumulative Oxygen Uptake for Propylene Glycol dinitrate (PGDN) and Aniline in a soil slurry reactor with 10% soil suspension.

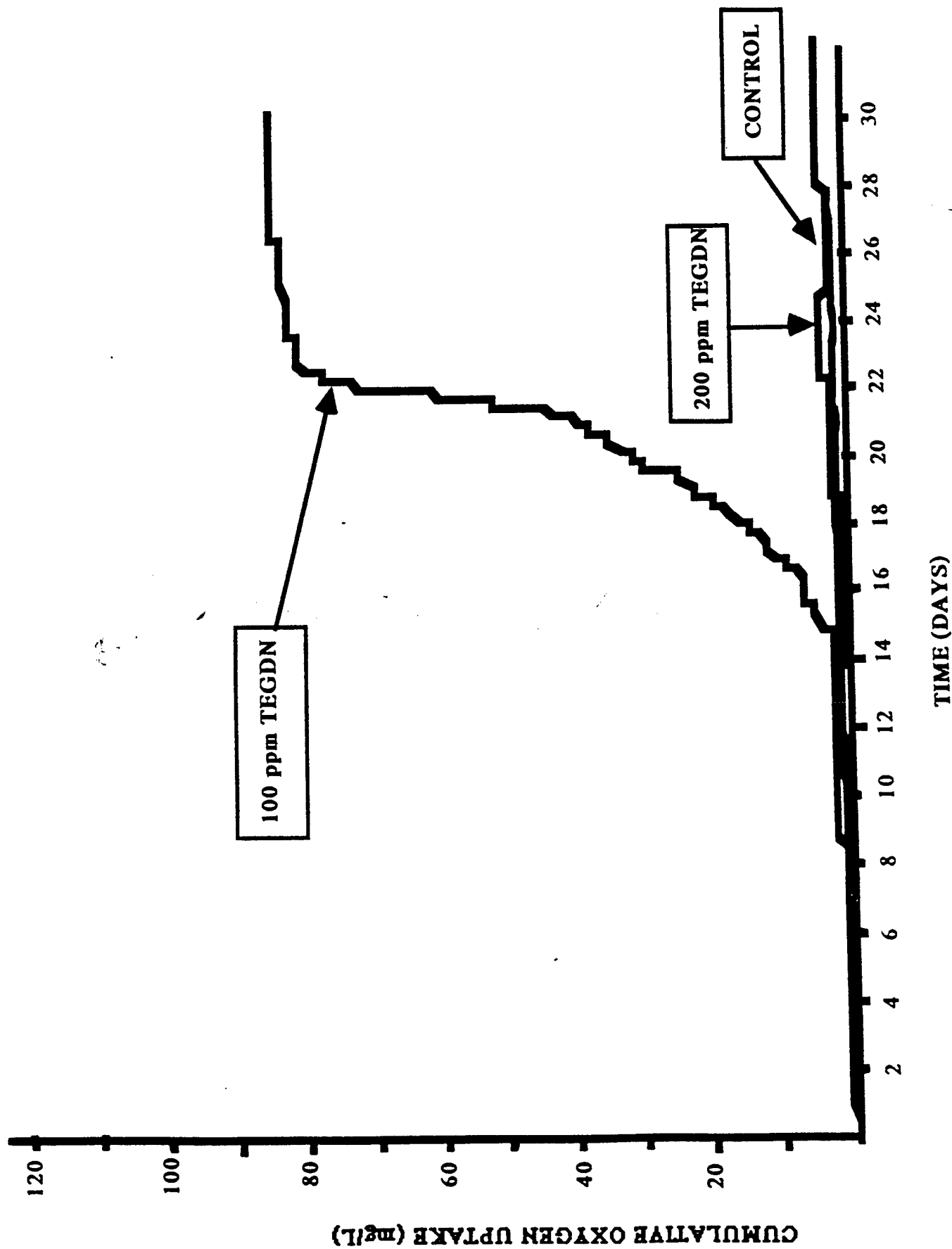


Figure 13. Cumulative Oxygen Uptake for Triethylene glycol dinitrate (TEGDN) in a soil slurry reactor with 10% soil suspension and initial TEGDN concentrations of 100 mg/L and 200 mg/L.

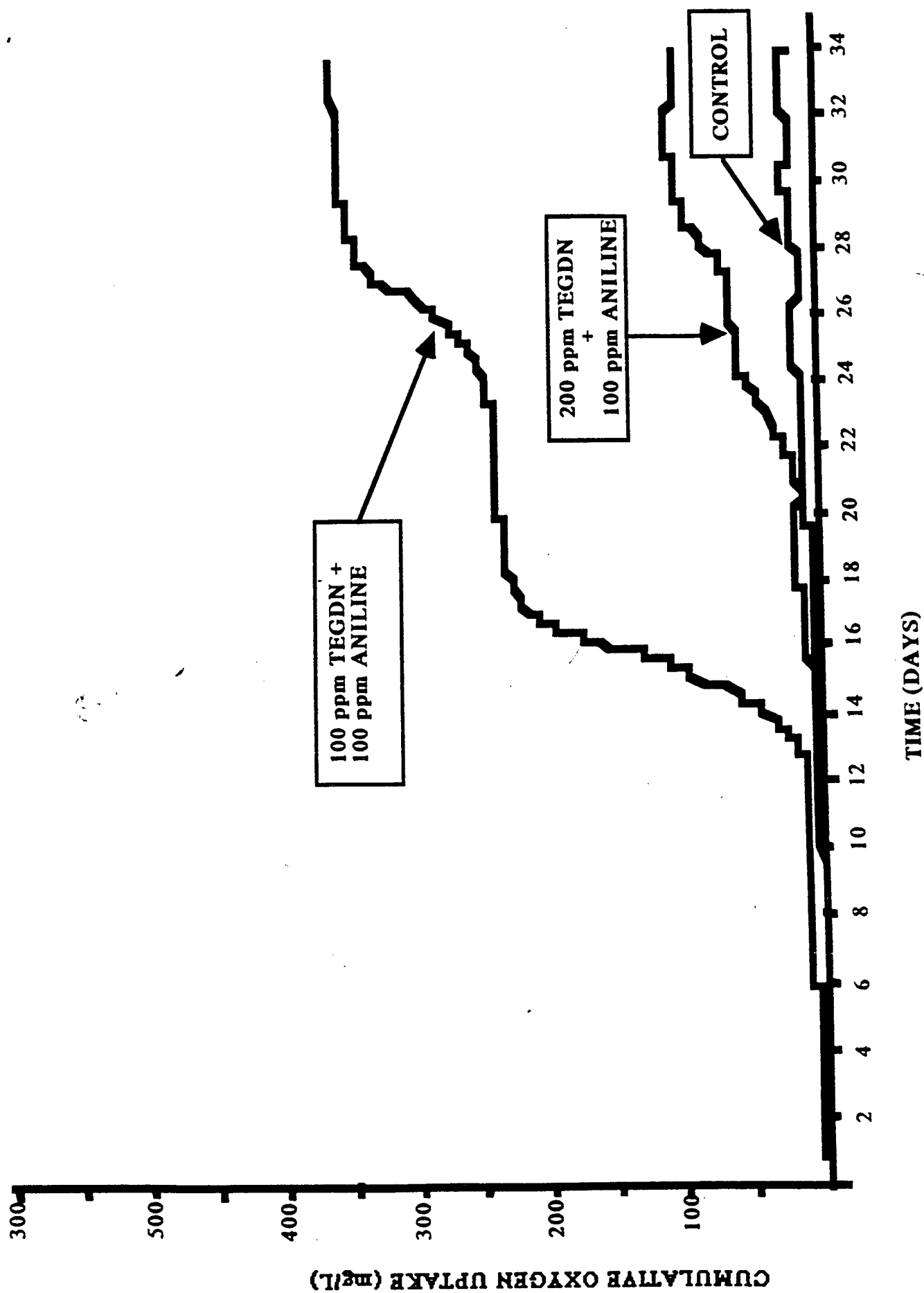


Figure 14. Cumulative Oxygen Uptake for Triethylene glycol dinitrate (TEGDN) and Aniline in a soil slurry reactor with 10% soil suspension.

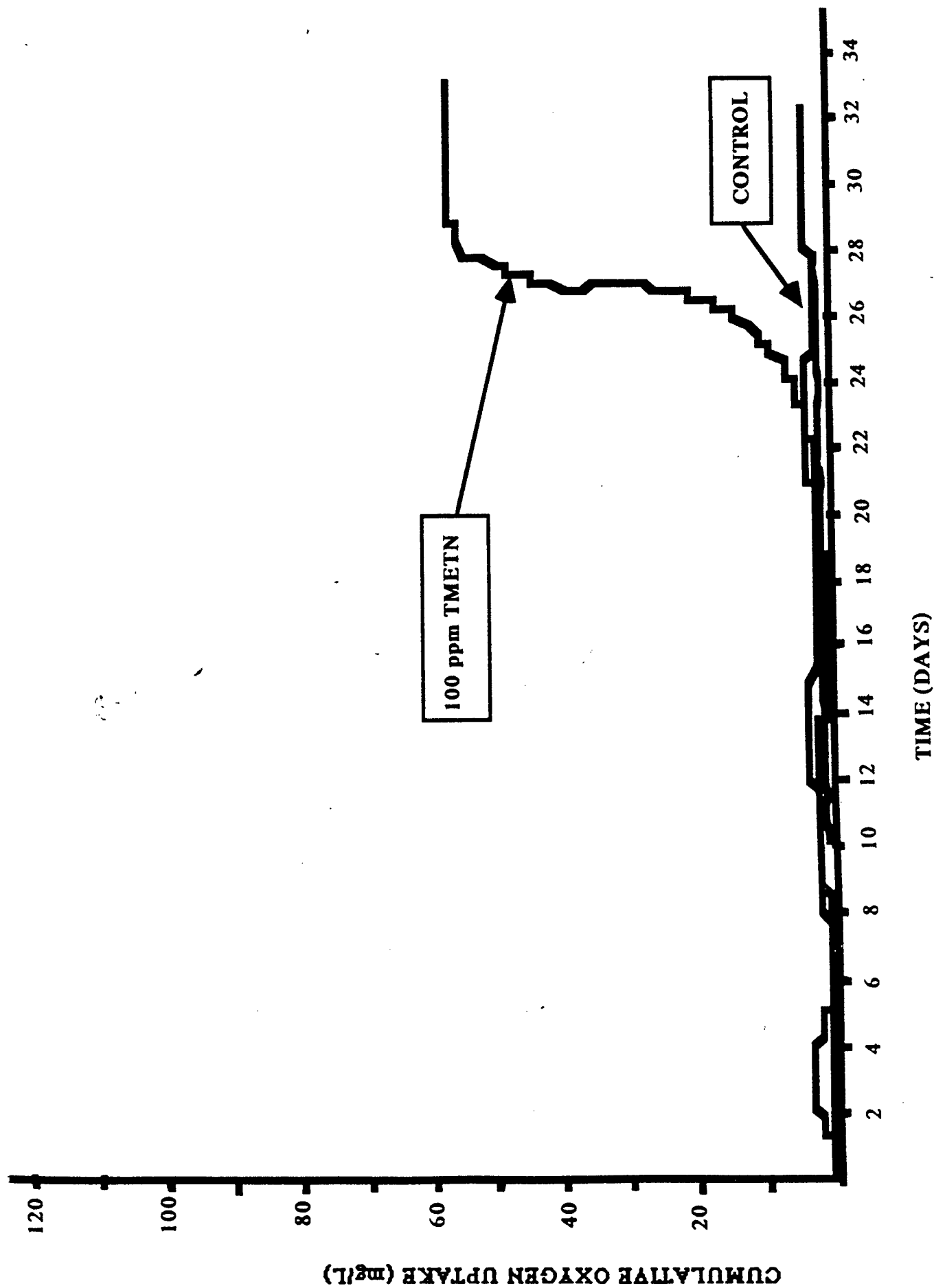


Figure 15. Cumulative Oxygen Uptake for Trimethyloethane trinitrate (TME TN) in a soil slurry reactor with 10% soil suspension and initial TME TN concentrations of 100 mg/L.

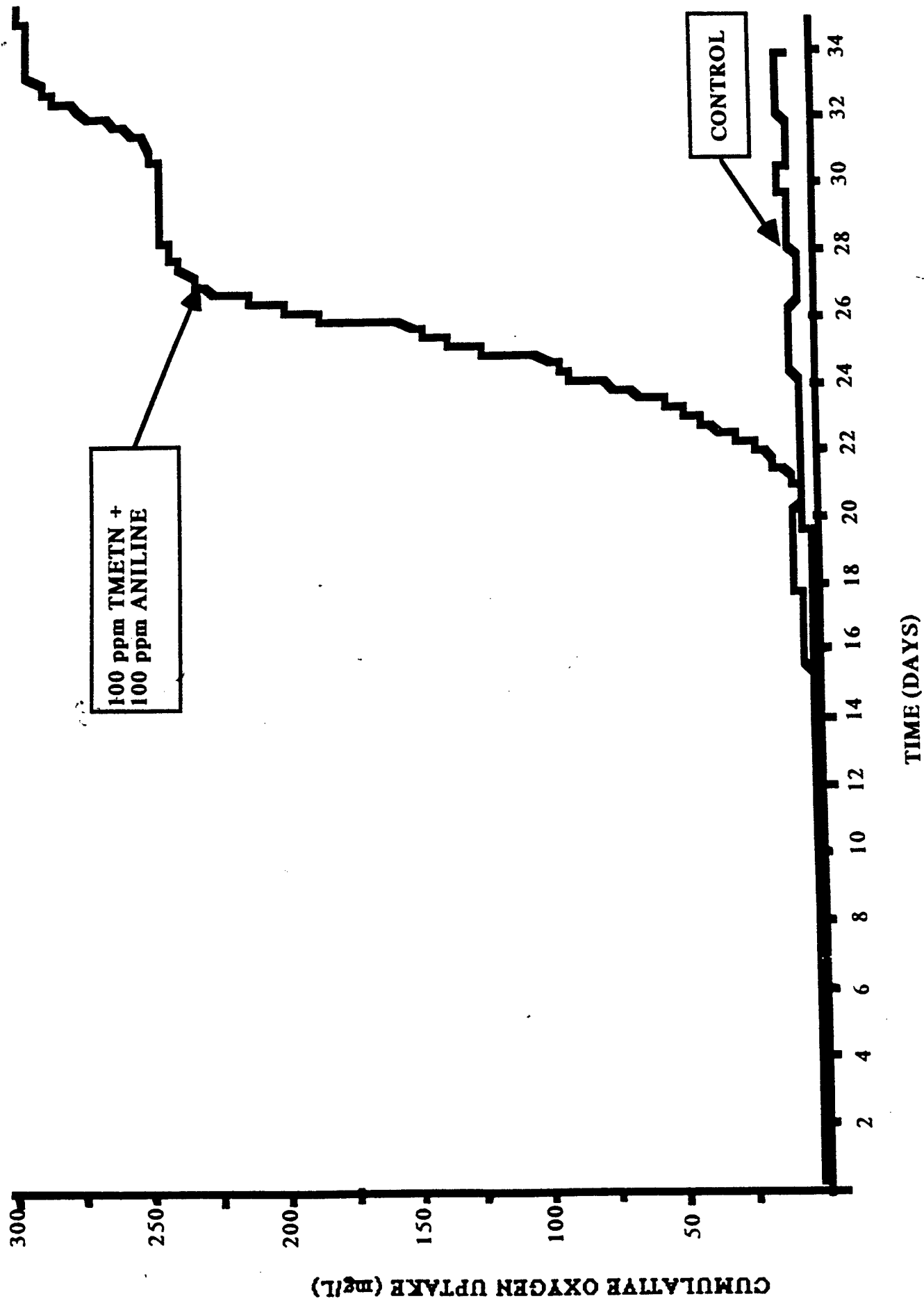


Figure 16. Cumulative Oxygen Uptake for Trimethyloethane trinitrate (TMETN) and Aniline in a soil slurry reactor with 10% soil suspension.

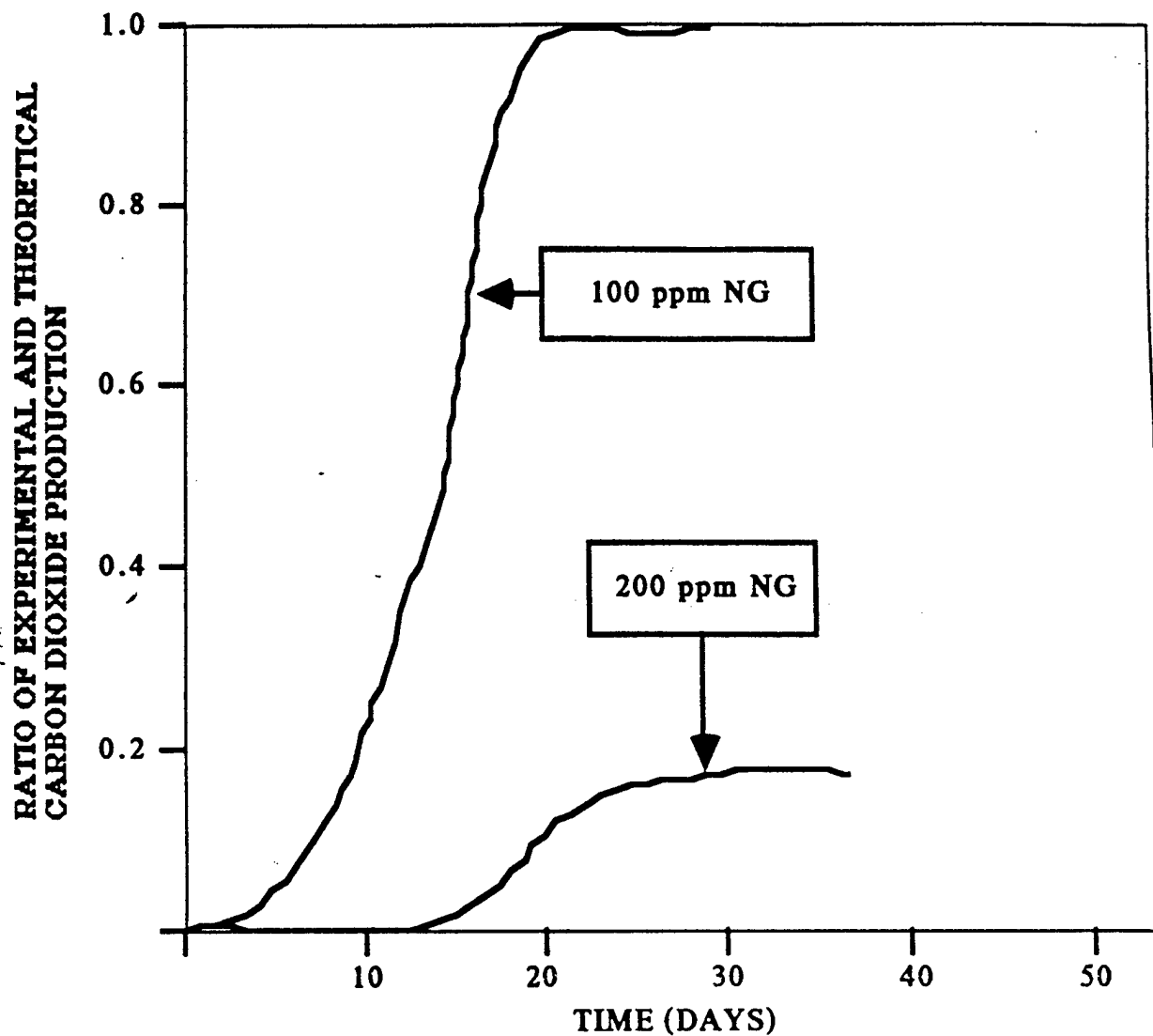


Figure 17. Cumulative carbon dioxide production as a ratio with the theoretical carbon dioxide amount versus time for Nitroglycerine (NG) at initial concentrations of 100 mg/L and 200 mg/L.

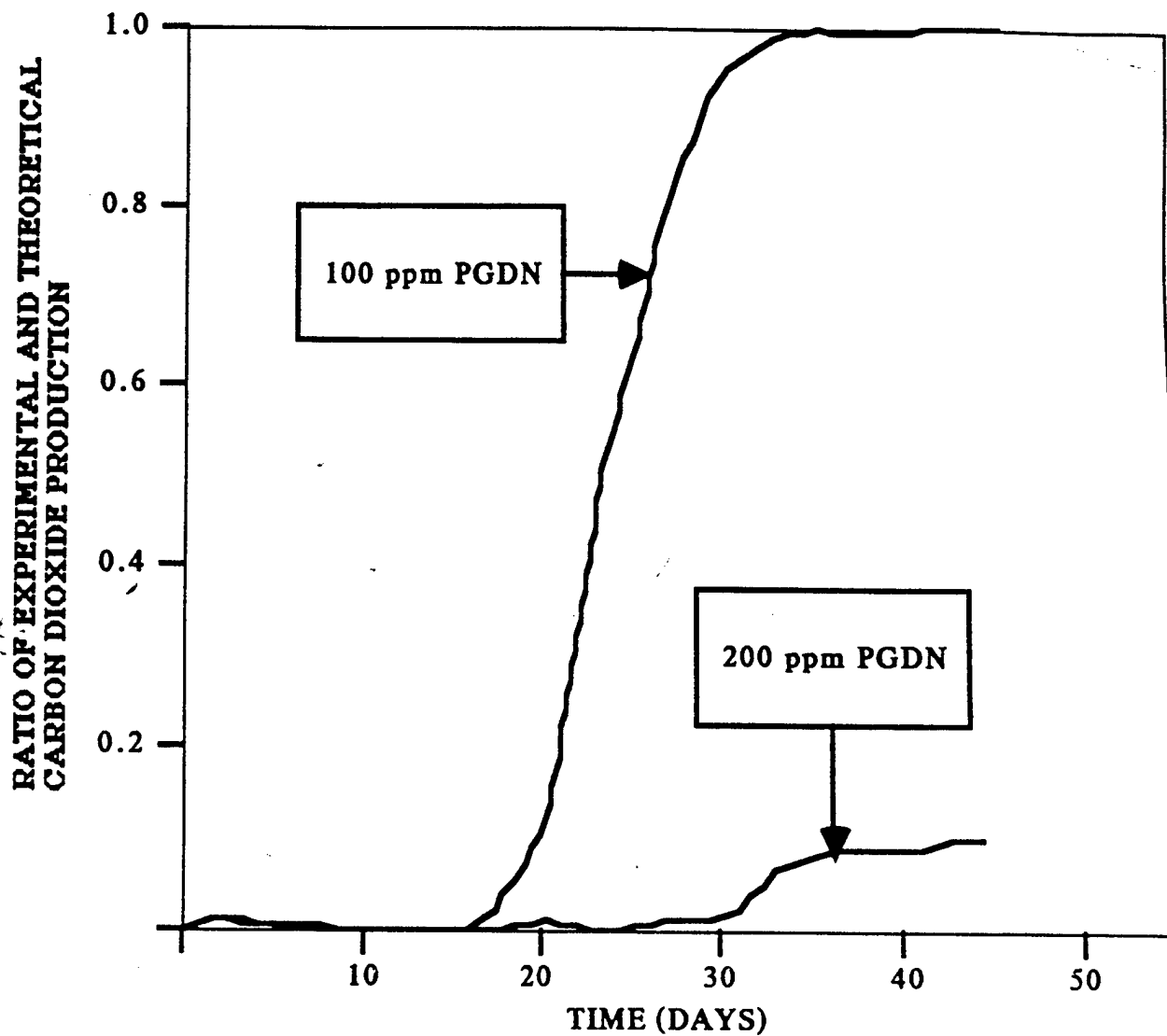


Figure 18. Cumulative carbon dioxide production as a ratio with the theoretical carbon dioxide amount versus time for Propylene Glycol dinitrate (PGDN) at initial concentrations of 100 mg/L and 200 mg/L.

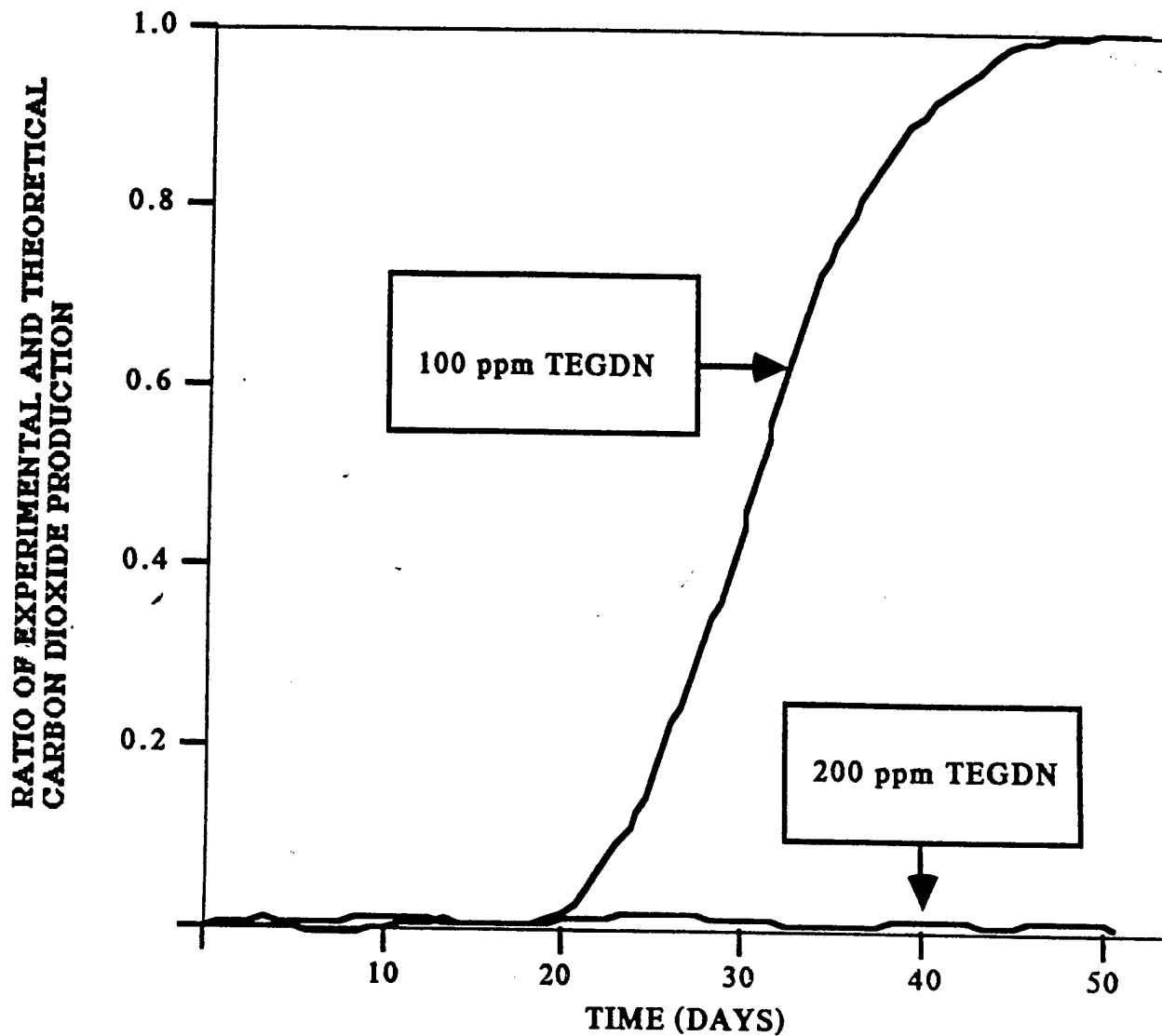


Figure 19. Cumulative carbon dioxide production as a ratio with the theoretical carbon dioxide amount versus time for Triethylene dinitrate (TEGDN) at initial concentrations of 100 mg/L and 200 mg/L.

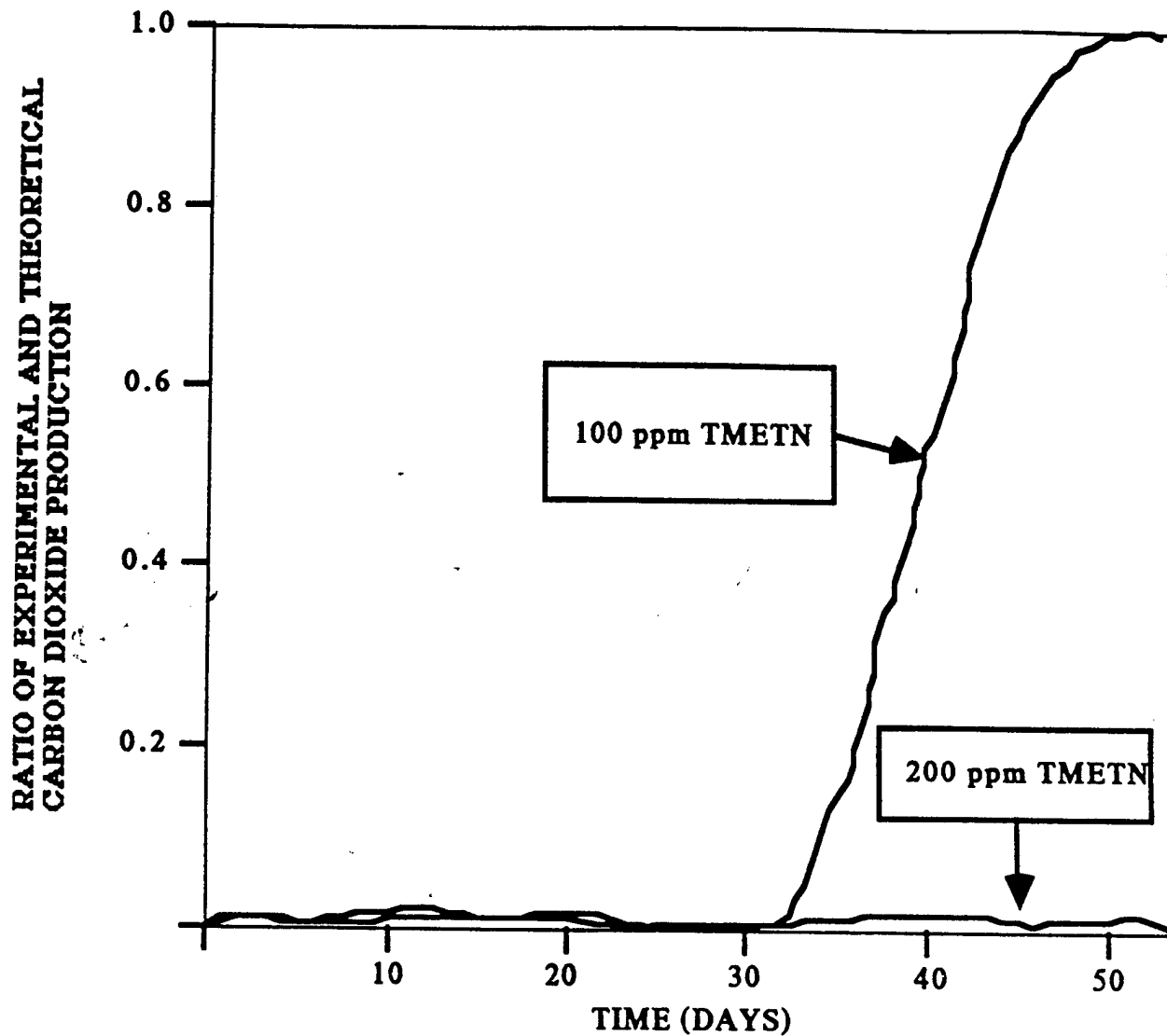


Figure 20. Cumulative carbon dioxide production as a ratio with the theoretical carbon dioxide amount versus time for Trimethyloethane trinitrate (TMETN) at initial concentrations of 100 mg/L and 200 mg/L.